THE IMMUNOREGULATORY ROLE OF NATURAL KILLER (NK) CELL DERIVED IL-10 DURING MICROBIAL INFECTIONS

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ABSTRACT

Natural Killer (NK) cells, lymphocytes of the innate immune response, play a vital role in controlling infections and in tumor surveillance. NK cells provide protection by direct cytolysis of infected cells and by the production of pro-inflammatory cytokines such as, IFN-γ and TNF-α. Notably, NK cells have recently been identified to regulate the immune response by producing the anti-inflammatory cytokine IL-10. Several other cells can produce IL-10 during infections, however NK cell derived IL-10 can be critical in regulating immune response during early phases of infection and therefore protecting the host from excessive immunopathology. Although the regulatory role of NK cells seems to be plausible, the physiological relevance of NK cell mediated immune regulation during infections has not been demonstrated in detail.

To investigate the immunoregulatory function of NK cells, I used Murine Cytomegalovirus (MCMV) infection induced by a high dose challenge and demonstrated that NK cells are a major IL-10 producer during acute stage of the infection. To elucidate the role of NK cell derived IL-10 during infections, I generated NK cell specific IL-10 knockout, *NKp46*^{iCre} × *Il-10*$^{flox/flox}$ *mice* (*NK-Il-10-/-*) by crossing *Il-10*$^{flox/flox}$ mice with mice expressing *Cre* recombinase exclusively under the NK cell specific promoter, NKp46 (*NKp46*^{iCre} knock-in mice). My results indicated that *Cre* mediated *Il-10* genomic deletion occurred predominantly in NK cells but not in NKT, T and B cells. Enriched NK cells from *NK-Il-10-/-* mice failed to produce IL-10 upon *ex vivo* IL-2/IL-12 stimulation. Furthermore, histological analysis of the colon indicated that *NK-Il-10-/-* mice are free from aberrant inflammation. During sustained MCMV infection, significantly higher production of IFN-γ

by CD8+ T cells of *NK-Il-10-/-* mice in salivary glands indicates that NK cell derived IL-10 contributes to the establishment of the immune suppressive environment in the organ. *NK-Il-10-/-* mice also demonstrated increased susceptibility to acute *Listeria monocytogenes* (LM) infection based on enhanced body weight loss. Taken together, I have successfully generated *NK-Il-10-/-* mice that lack the *Il-10* gene exclusively in NK cells. The *NK-Il-10-/-* mouse can be used as an ideal model to dissect the immunoregulatory role of NK cells during various microbial infections and tumorogenesis.

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LIST OF ABBREVIATIONS

μg	microgram
μl	microliter
μm	micrometer
°C	Degrees Celsius
ACK	Ammonium-chloride-potassium
ActA	Actin-assembly-inducing protein
ADCC	Antibody Dependent Cellular Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
ANG2	Angiopoietin 2
APC	Antigen Presenting Cells
BFA	Brefeldin A
BHI	Brain Heart Infusion
BMT	Bone Marrow Transplant
bp	Base pairs
CBA	Cytometric Bead Assay
CC	Chemokines
CCL	Chemokine Ligand
CD	Cluster of Differentiation
CFU	Colony Forming Units
CM	Conditioned Media
CMV	Cytomegalovirus

CNS	Central Nervous System
Cre	*C*yclization *re*combinase
CTL	Cytotoxic Lymphocyte
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
CTLR	C-type lectin-like receptor
DAP	DNAX Activation protein
DCs	Dendritic Cells
DMEM	Dulbecco's Modified eagle Medium
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr virus
FBS	Fetal Bovine Serum
Foxp3	Forkhead boxP3
GC	Guanine-Cytosine
gps	Glycoproteins
h	hour
H&E	Hematoxylin and Eosin
HA	Hemagglutinin
HBV	Hepatitis B Virus
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSCT	Hematopoietic Stem Cell Transplantation
HSV-1	Herpes Simplex Virus-1

IBD	Inflammatory Bowel Disease
IFN-γ	Interferon-γ
IgSF	Immunoglobulin like superfamily
IR	Internal Repeats
IRAK	Interleukin-1 Receptor Associated Kinase
IRF	IFN Regulatory Factor
ITAM	Immuno-receptor Tyrosine-based Activation Motifs
ITIM	Immuno-receptor Tyrosine-based Inhibition Motifs
Kbp	Kilo basepair
KDa	Kilo Daltons
KIR	Killer Ig-like Receptor
KLRG1	Killer cell Lectin- like Receptor G1
LAMP1	Lysosomal-Associated Membrane Protein 1
LCMV	Lymphocytic Choriomeningitis Virus
LLO	Listeriolysin O
LM	*Listeria monocytogenes*
LM	Low-melting
LoxP	Locus of X-over of P
M	Molar
Mϕ	Macrophage
mC-BP	mCP binding protein
MCMV	Murine Cytomegalovirus
MCP	Major Capsid protein

mCP	Minor Capsid Protein
MHC	Major Histocompatibility Complex
MHV	Mouse Hepatitis Virus
min	minutes
MIP-1	Macrophage inflammatory protein 1
ml	milliliter
mm	millimeter
NCR	Natural Cytotoxicity triggering Receptor
NF-κB	Nuclear Factor Kappa-light-chain- enhancer of activated B cells
NK	Natural Killer
NKC	NK gene Complex
NOS2	Nitric Oxide Synthase type 2
ORFs	Open Reading Frames
OV	Orf poxvirus
p.i.	Post Infection
PAMPs	Pathogen Associated Molecular Patterns
PCR	Polymerase chain reaction
pDCs	Plasmacytoid Dendritic Cells
PFA	Paraformaldehyde
PFU	Plaque Forming Units
pg	pico gram
PLGF	Placental Growth Factor
prf-/-	Perforin deficient

PRRs	Pattern Recognition Receptors
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
s	Seconds
SCID	Severe Combined Immunodeficiency
SCP	Smallest Capsid protein
SH-2	Src homology-2
SLE	Systemic Lupus Erythematosus
STAT	Signal Transducer and Activator of Transcription
TGF-β	Transforming Growth Factor-beta
tiger	Interleukin-*t*en-*i*res-*g*fp-*e*nhanced-*r*epoter
TIR	Toll/IL-1R
TLRs	Toll- Like Receptors
TNF-α	Tumor Necrosis Factor-α
TRAIL	TNF-related apoptosis-inducing ligand
T_{regs}	Regulatory T cells
TRIF	TIR domain-containing adaptor–inducing IFN-β
TRS	Terminal Repeat Sequences
UL	Unique Long
US	Unique Short
VEGFC	Vascular Endothelial Growth Factor C
VZV	Varicella Zoster Virus

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1. INTRODUCTION

The immune system has evolved over time to protect the host from pathogens. During infections, various immune cells are activated to exert their allotted roles in order to combat the evading microbes. The recognition of a pathogen by immune cells triggers a cascade of antimicrobial mechanisms that ultimately leads to clearance of the pathogen (Medzhitov, 2007). NK cells are the lymphocytes of innate immune response that play an important role in controlling viral infections (Biron et al., 1999; Lee et al., 2007), and also tumor surveillance (Pegram et al., 2011; Vivier et al., 2012). NK cells mediate protection by direct cytolysis of target cell and production of pro-inflammatory cytokines, such as IFN-γ and TNF-α. NK cell function is controlled by vast array of activating and inhibitory receptors expressed on their surface. NK cells, upon stimulation via their activating receptors, induce target cell lysis, cytokine production and may undergo proliferation (Lodoen and Lanier, 2006). Besides NK cells, another lymphocyte population critical in clearing infections in hosts is T cells. T cells undergo clonal expansion in an antigen-specific manner upon recognition of a virus-infected cell and subsequently result in killing of infected cells via cytotoxic activity. T cells also provide long-term immunity against the pathogens through memory cell production (Broere, 2011).

However, the immune response exerted by either NK cells or T cells during microbial infections must be tightly regulated to maintain sufficient levels of immune response for pathogen elimination while avoiding excessive immunopathology (Hedrich and Bream, 2010). IL-10 is a potent immunosuppressive cytokine that ameliorates excessive host-damaging immune responses. IL-10 interferes with the expression of major histocompatibility complex (MHC) and co-stimulatory molecules on antigen presenting cells

(APCs) like, macrophages and dendritic cells (DCs), and also limits the production of pro-inflammatory cytokines (Joss et al., 2000; Moore et al., 2001). Various immune cell subsets like dendritic cells, macrophages, etc., produce IL-10, but regulatory T cells (T_{reg}) are the major producers of this cytokine (Joss et al., 2000). Notably, there have been studies showing IL-10 production by NK cells (Brockman et al., 2009; Chakir et al., 2001; Deniz et al., 2008; Lee et al., 2009; Maroof et al., 2008; Perona-Wright et al., 2009). This observation was rather surprising because NK cells have been known cells capable of producing pro-inflammatory cytokines such as IFN-γ and TNF-α. During MCMV infection of immunocompromised mice, NK cells are shown to be major producer of IL-10 in the acute phase of infection. In this model, the NK cell derived IL-10 acts to limit the magnitude of the CD8+ T cell response in immunocompromised perforin-deficient (*Prf-/-*) mice (Lee et al., 2009). The objective of my project was to identify whether such a NK cell-mediated regulatory pathway also exist in immunocompetent mice by generating NK cell specific IL-10 knockout mice (*NK-Il-10-/-*) and to elucidate the regulatory role of NK cell during Murine Cytomegalovirus (MCMV) and *Listeria monocytogenes* (LM) infection using this mouse.

1.1. Pathogen: Cytomegalovirus

1.1.1. General introduction

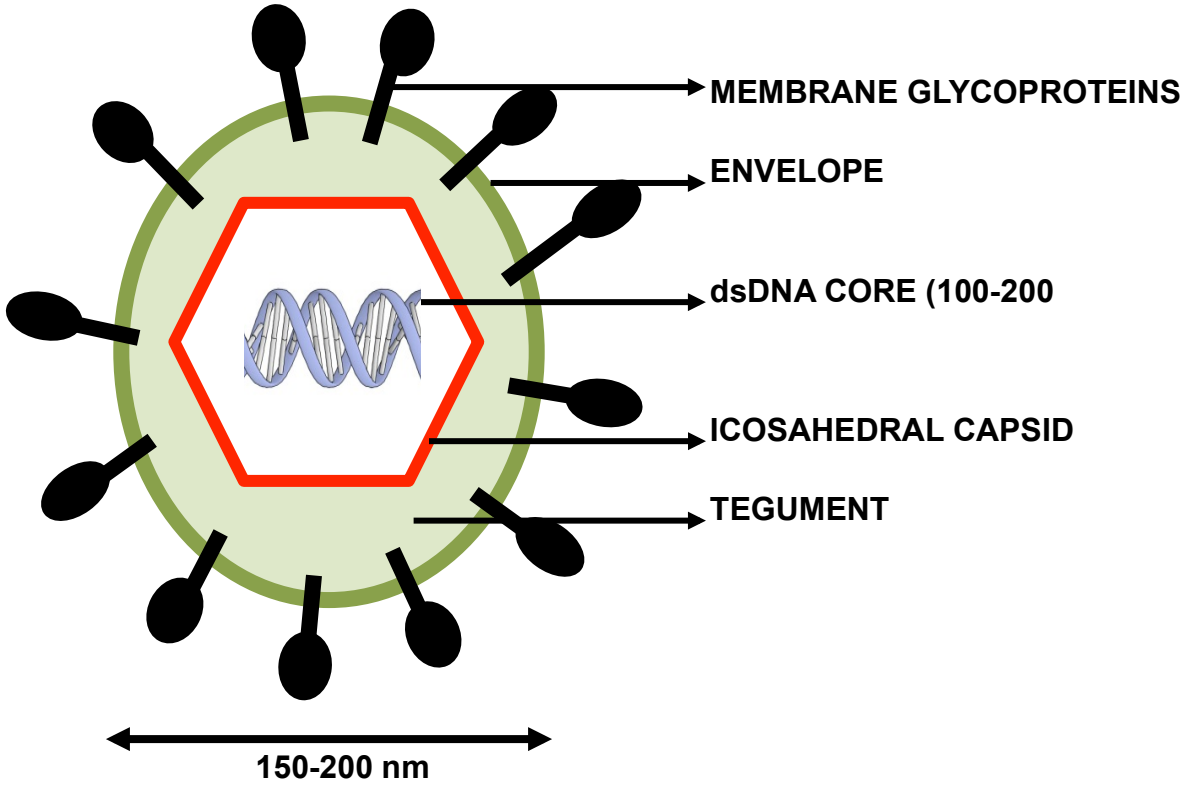
The Cytomegalovirus (CMV) belongs to the herpesviridae family. Within herpesviridae, CMV is classified under subfamily betaherpesvirinae along with two other groups, muromegalovirus and roseolovirus (Roizmann et al., 1992). The virus used to be called cytomegalic inclusion virus and/or salivary gland virus until Weller proposed the common name cytomegalovirus in 1957 (Craig et al., 1957). CMV is closely related to

members of other herpesvirus subfamilies like Varicella-Zoster Virus (VZV) and Herpes Simplex Virus-1 (HSV-1). Like other herpesviruses, CMV is also capable of establishing latent infection in the host (Weller, 1971). CMV infection can be identified by its characteristic biological attributes: induction of cytomegalia and formation of cytoplasmic inclusion bodies (Weller, 1971). Generally CMV infection is asymptomatic but can be detrimental in an immunocompromised host.

1.1.2. Structure

CMV is a double stranded DNA virus with an approximate genome size of 100-200 kbp. The GC content of CMV ranges from 31%- 75% (Roizmann et al., 1992). Its genome consists of two domains: unique long (UL) and unique short (US). Both of these domains are flanked by terminal repeated sequences (TRL and TRS) on one side and internal repeats (IRL and IRS) on the other side (Bankier et al., 1991). The genome of human CMV (HCMV) is recognized as the largest among the herpesvirus family with an estimated 160-250 open reading frames (ORFs) (Chee et al., 1990). Functional profiling of the genome has revealed that roughly 45 genes dispensable for virus growth are highly conserved across herpesviruses (Dunn et al., 2003). The CMV particle is approximately 200 nm in diameter and made up of four structural elements: core, capsid, tegument and envelope as shown in **Figure 1.1**. The large double stranded DNA forms the core of the virus enclosed by an icosahedral capsid. The core and capsid together are known as the nucleocapsid. The CMV capsid structure closely resembles that of HSV-1. The capsid has a diameter of ~110 nm and consists of 162 capsomeres and 320 triplexes located between capsomeres (Gibson, 2008). The capsid is made of four different proteins: major capsid protein (MCP), minor capsid protein (mCP), mCP binding protein (mC-BP) and the smallest capsid protein (SCP).

Figure 1.1 Schematic cartoons depicting complete structure of CMV. The CMV structure is composed of four elements: core, capsid, tegument and envelope.


MEMBRANE GLYCOPROTEINS
ENVELOPE
dsDNA CORE (100-200
ICOSAHEDRAL CAPSID
TEGUMENT
150-200 nm

The tegument layer of CMV lies between the genome-containing nucleocapsid and the viral envelope. Electron microscopic visualization of the CMV virus has suggested an amorphous structure of tegument proteins (Trus et al., 1999). Of the total proteins found in infectious virus, roughly half of them form the tegument, and the rest constitute the capsid and envelope proteins. In addition to viral proteins, the tegument layer also consists of roughly 70 cellular proteins, and function of these proteins is largely unknown (Varnum et al., 2004). The tegument proteins are released inside the host cell cytoplasm after fusion of viral envelope and host membrane. They assist in viral entry, function to regulate the gene expression, and participate in immune evasion. These proteins also direct assembly and release of viral progeny. Thus, viral tegument proteins play critical roles in very early and later phases of the CMV replication cycle (Kalejta, 2008).

The lipid bilayer envelope of CMV encloses the nucleocapsid and tegument to form a fully functional infectious virus particle. The envelope glycoproteins are obtained from the host cell membrane. Additionally, it contains nine virally encoded glycoproteins such as gB (UL_{55}), gM (UL_{100}), gH (UL_{75}), gL (UL_{115}), gO (UL_{74}), gN (UL_{73}), gP48 (UL_{4}), gpTRL_{10} and UL_{33} (Britt and Mach, 1996; Varnum et al., 2004). The glycoprotein gB is highly conserved among herpesviruses and is a dominant B-cell antigen in CMV infected animals and humans (Rawlinson et al., 1996). The virus gains entry to the host cell by fusion of viral envelope with the host cell membrane mediated by viral glycoproteins (Gibson, 2008)

1.1.3. CMV pathogenesis

CMV can replicate inside epithelial cells, endothelial cells, smooth muscle cells, mesenchymal cells, granulocytes and monocyte-derived macrophages (Plachter et al., 1996; Sinzger et al., 1996). Histopathological and immunohistochemical techniques have been used

to study the pathogenesis of CMV. CMV first establishes infection in epithelial cells of the respiratory and upper alimentary tracts. The virus replication in the development of characteristic cytoplasmic inclusion bodies on the erythematous base (Landolfo et al., 2003). Additionally, Leukocytes and vascular endothelial cells help in virus dissemination during acute infection. Furthermore, virus-encoded chemokines induce chemotaxis of neutrophils and monocytes resulting in viral spread from the site of infection to other organs of the body (Penfold et al., 1999). Besides acute infection, CMV is well known for developing latent infection in various organs, for example, salivary glands, breast, kidney, endocervix, seminal vesicles and peripheral blood leukocytes (Landolfo et al., 2003). The latent infection results in viral release from involved organs and is thereby a common cause of chronic viral transmission.

1.1.4. CMV infection in humans

HCMV is a widespread pathogen among humans. About 60-80% of individuals acquire HCMV by the age of 40. Generally this infection is asymptomatic; it may, however, lead to fatal consequences in immunocompromised individuals. Clinical manifestations of HCMV infection include mononucleosis, persistent fever, myalgia, headache, cervical lymphadenopathy and splenomegaly. Uncommon complications associated with HCMV infection include pneumonia, myocarditis, hemolytic anemia, retinitis, Central nervous system (CNS) involvement and peripheral neuropathy (Landolfo et al., 2003). Following infection, virus is secreted in body fluids like urine, saliva, tears, breast milk, semen and cervical discharges facilitating both vertical and horizontal transmission from the host (Pass, 1985). Congenital infection of HCMV in babies can result in birth defects. A mother can transmit HCMV infection to baby via placenta, during delivery and also while breast-

feeding. In some rare cases, congenital infection can lead to serious consequences, including, CNS damage, loss of hearing capacity and developmental disabilities (Landolfo et al., 2003).

HCMV is one of the leading opportunistic pathogens for immunocompromised individuals. Its impact has become apparent in patients with organ transplants, patients on immunosuppressive therapies and AIDS patients (Pass, 1985). Individuals with bone marrow transplants (BMT) and AIDS often develop severe HCMV syndrome, associated with pneumonitis. Dissemination of CMV in the gut may be asymptomatic in healthy individuals but may result in severe ulceration in patients on immunosuppressive therapy. CMV can infect the CNS of AIDS patients, leading to similar complications as reported during congenital infections. HCMV mediated immunosuppressive syndrome in the post-transplant period can also result in increased susceptibility to bacteria, fungi and protozoa resulting in increased morbidity. CMV induced viremia is a major cause of morbidity and mortality following hematopoietic stem cell transplantation (HSCT) (Boeckh et al., 2003). Taken together, HCMV is a significant opportunistic pathogen in immunocompromised individuals.

1.1.5. Mouse model of CMV

The strict species specificity of CMV precludes experimental infections of animals with HCMV. Therefore, the mouse adaptation of CMV (MCMV) is an equivalent model system to study the pathogenesis of this virus and answer questions that cannot be easily addressed by clinical research. Both MCMV and HCMV are similar in terms of virion structure, pathogenesis and cell biology (Shellam, 2007). Thus, mouse infection with CMV recapitulates most of the clinical manifestations observed during virus-induced human diseases. The genomes of both viruses are co-linear, with high similarity at genetic and nucleotide composition levels. MCMV genes share around 78% similarity to HCMV genes.

The sequences for similar genes are all located in the central region of the genome, whereas sequences near the ends of the genome are unique to particular CMV strains and mostly encode for glycoproteins (gps) and immune evasion genes (Rawlinson et al., 1996).

Both viruses establish latent infection that can be reactivated upon immunosuppression to cause disseminated and often fatal disease. Major cell types and organs infected by both viruses, the course of infection, and the type of pathology observed are essentially identical, with the notable exception that MCMV does not cross the placenta (Shellam, 2007). Moreover, different mouse strains differ in their susceptibility to MCMV infection, indicating an important role played by host factors in controlling susceptibility (Smith et al., 2008). Therefore, the experimental model of mouse infection with MCMV is an excellent tool to study the course of virus infection and to dissect the components of the immune response that are important for mediating resistance against MCMV and HCMV infection.

1.2. Innate immune response

The immune system is composed of two major branches: innate- or non-specific-immune response, and adaptive- or specific-immune response. Although both innate and adaptive immune systems function to protect against invading pathogens, they differ in a number of ways. First, the adaptive immune system requires some time to get activated against an invading organism, whereas the innate immune system is quick. Second, the adaptive immune system is antigen specific and reacts only to the organism that induced the response. In contrast, the innate system is not pathogen specific. Finally, the adaptive immune system confers immunological memory whereas the innate immune response lacks memory generation (Travis, 2009). The importance of innate immunity can be appreciated by the fact that development of a specific adaptive immune response takes days to weeks,

whereas the innate immune system protects the host during the time between microbe exposure and initial adaptive responses.

The innate immune system has three major components: the first component comprises physical or chemical barriers, for example skin, epithelial linings and gastric pH. The skin and epithelial linings act as barriers by preventing pathogen entry to the host cells. Likewise, an acidic environment inside the stomach renders protection against pathogens that are swallowed and also the intestine's digestive juices and bile are potentially destructive to microbes. The second component includes soluble compounds such as serum, mucus, defensins, etc. The mucus prevents the adherence of pathogens to the epithelial lining of the lungs and gut. The mucous layer also contains antimicrobial substances like defensins that inhibit pathogen growth. The third component includes innate immune cells such as neutrophils, macrophages, dendritic cells (DCs), mast cells and NK cells. The activation of these cells is dependent upon the recognition of conserved sequences-pathogen-associated molecular patterns (PAMPs)-found on pathogen surface by pattern recognition receptors (PRRs) expressed on the cell surface (Medzhitov and Janeway, 2000). The PRRs mediated PAMP recognition initiates phagocytosis, opsonization, induction of apoptosis, activation of complement and pro-inflammatory cytokine signals. The immune cell populations, including their specific roles and modes of action, will be discussed briefly below.

1.2.1 Macrophages (MΦ)

Macrophages belong to the class of mononuclear phagocytes and are ubiquitously distributed cell populations responsible for basic processes like homeostasis and immune responses. They participate in innate immune responses against a variety of pathogens and in

adaptive immune responses by antigen processing and presentation. Macrophages express germ-line encoded PRRs that can recognize PAMPs (Keller et al., 1995). Among various PRRs identified to date, Toll-like receptors (TLRs) are the most extensively studied. TLRs are type I transmembrane proteins with an extracellular domain consisting of multiple leucine-rich repeat elements and a cytoplasmic Toll/IL-1R homology (TIR) domain. The extracellular domain recognizes the PAMPs, whereas the cytoplasmic domain activates the downstream signaling pathways. Upon recognition of a PAMP, TLRs recruit a set of TIR-containing adaptor molecules to transduce signals through MyD88, IRAK, NF-κB and mitogen activated protein kinases (MAPK) resulting in the expression of a variety of pro-inflammatory cytokines and chemokines and promotion of phagocytosis. (Kawai and Akira, 2006; Kawai and Akira, 2011).

Macrophages play an important role during viral infections. Since macrophages are in close contact with circulating blood, they are likely to be the first cell type infected by blood-borne viruses. Mϕ support viral replication during MCMV infection, but they also mediate an early innate responses. Activated macrophages migrate to the site of infection and initiate a cascade of antiviral responses. The macrophage-derived nitric oxide (NO) appears to be important in eliminating various viruses, including CMV. Together with DCs, Mϕ also act as APCs, resulting in activation of the adaptive immune response. For example, Mϕ activated by IFN-γ during infections can increase MHC expression and antigen processing and thereby resulting in increased antigen presentation to T cells (Hanson et al., 1999).

1.2.2 Dendritic Cells (DCs)

DCs are a critical cell population of the immune system that links innate and adaptive immune responses. The DCs are abundant in the skin, mucosal surfaces and in lymphoid organs. The stimulation of DCs during early phases of infection initiates their differentiation to professional APCs, capable of processing and presenting antigens in the context of MHC to naïve T cells. In addition to antigen presentation, DCs also produce myriad cytokines upon antigen exposure, including, IL-12 and interferons. These cytokines further activate other innate immune cells and also enhances T and B cell mediated adaptive immune responses (Steinman and Hemmi, 2006). Like other immune cells, DCs too express an array of surface receptors that are plays critical role in conferring DC mediated pathogen elimination and activation of adaptive immune response.

DCs can be classified into different subsets based on the expression of surface markers, such as Ly6G/C^{-}CD8α^{-}CD11b^{+} (CD11b^{+} DC), Ly6G/C^{-}CD8α^{+}CD11b^{-} (CD8α^{+} DC) and Ly6G/C^{+}CD8$\alpha^{-/+}$CD11b^{-} plasmacytoid DC (pDCs). Besides, these markers, DCs also express a variety of other cell surface C-type lectin receptors that mediate antigen uptake. Not much is known about the natural ligands of these receptors except DC-SIGN/CD209, a receptor that is able to recognize mannose and fucosyl residues on the surface of a variety of pathogens, such as HIV, CMV, Ebola virus, dengue virus and *Candida* species (Figdor et al., 2002). Like Mϕ, DCs employ TLRs to recognize microbial infections. Upon binding, they activate a signaling cascade leading to pro-inflammatory cytokine production. In particular, binding of MCMV infected cells to TLR9 expressed by pDCs result in the production of interferons and IL-12 that, in turn, mediate protection against MCMV (Takeda and Akira, 2005). Moreover, the type I interferons (IFNα/β) produced by DCs regulate the induction of

adaptive immune responses and also limits viral replication and IL-12 production. (Dalod et al., 2003).

1.2.3 NK cells

NK cells are so called because of their propensity to kill infected or transformed cells without any prior antigen stimulation (Kiessling et al., 1976). They are lymphocytes of the innate immune response that play a vital role during viral infections, especially herpesviruses infection (Arase et al., 2002; Bekiaris et al., 2008; Lee et al., 2007) and tumor surveillance (Smyth et al., 2002). Individuals without NK cells, or lacking normal NK-cell activity, experience persistent and life-threatening infections of normally innocuous viruses, particularly those of the herpesvirus and papillomavirus families (Orange, 2006). Also, in mice lacking NK cells, increased viral titer is observed in spleen and liver following mouse hepatitis virus (MHV), MCMV and vaccinia virus infection, suggesting a link between enhanced host susceptibility and NK cells (Bukowski et al., 1983; Welsh et al., 1990).

While NK cells are not generally thought to play role in bacterial infections, but it provide numerous ligands for TLRs and other PRRs and induce very characteristic inflammatory responses. Thus, there is considerable potential for indirect activation of NK cells during bacterial infection and for NK cell-derived IFN-γ to enhance phagocytosis of extracellular bacteria or infected host cells by macrophages (Horowitz et al., 2011). Not much is known about direct activation, but indirect activation of NK cells by bacteria is well described. For example, NK cell activation by LM, following binding of listeriolysin O (LLO) to TLR2 is mediated through the production of IL-1β, IL-12, IL-18, and TNF-α from

macrophages and DCs (Horowitz et al., 2011; Humann and Lenz, 2010). In addition, NK cells can also modulate the antigen presentation function of both DCs and Mϕ, and thereby promoting the generation of subsequent adaptive immune response (Degli-Esposti and Smyth, 2005). Furthermore, NK cells are also the predominant population among immune cells in a mother uterus, indicating their importance during pregnancy. The NK cells secrete cytokines during implantation that are involved in angiogenesis and vascular stability, for example, vascular endothelial growth factor C (VEGFC), placental growth factor (PLGF) and angiopoietin (ANG2) (Li et al., 2001; Moffett-King, 2002).

Recently, NK cells have been shown to mediate Ag-specific recall responses in several model systems. Although NK cells do not rearrange the genes encoding their activating receptors, they undergo a clonal-like expansion during virus infection, generate long-lived memory cells, and mediate more efficacious secondary responses against previously encountered pathogens (Sun et al., 2011). A recent study demonstrated that NK cells mediate contact hypersensitivity responses to chemical haptens in mice lacking T and B cells. These responses persisted for >4 weeks, and were only elicited by the hapten to which mice were originally exposed and not by a different hapten suggesting that NK cells can persist for longer periods and have the potential to contribute alongside memory T and B cell responses during subsequent encounters with the same pathogen (O'Leary et al., 2006). Taken together, Mϕ, DCs and NK cells are important cell populations of the innate immune response. They mediate protection against pathogens by phagocytosis and the production of a myriad of cytokines. The cytokines produced by these innate immune cells interferes with pathogen replication; thereby limiting the pathogen spread and also helps to shape the appropriate adaptive immune response.

1.3. NK cell receptors

NK cells express germ-line encoded receptors that do not undergo somatic rearrangement like T and B cell receptors. Broadly, NK cell receptors can be classified as activating or inhibitory. The balance between activating and inhibitory signals determines the outcome of NK cell function (Lodoen and Lanier, 2006). There are three major NK cell receptor families: killer Ig-like receptor (KIR) family in humans, Ly49 family in mice and NKG2 lectin-like receptor that are found in both humans and mice (Lanier, 1998).

1.3.1. NK inhibitory receptors

NK cells differentiate between self and non-self based on class I MHC expression. The MHC-I is expressed on all nucleated self cells, and its binding to NK cell inhibitory receptors results in suppression of NK cell cytotoxicity. On the other hand, the infected or transformed cells tend to down-modulates MHC-I expression in order to avoid recognition by T cells, but an absence or lower expression of MHC-I render them highly susceptible to NK cell killing. This phenomenon of recognition and elimination of cells lacking self-MHC I molecules by NK cells is termed the "missing self hypothesis" (Ljunggren and Karre, 1990). Since all normal cells ubiquitously express MHC class I, missing-self adequately explains protection against NK cell auto-aggression. NK cells have the potential to cause significant damage to normal self cells unless controlled by self-tolerance mechanisms. NK cells normally acquire self-tolerance through the interaction of their inhibitory receptors with the cognate MHC-I molecule, a process known as licensing (Jonsson and Yokoyama, 2009). The licensing hypothesis proposes that an NK cell must engage self-MHC in order to be

responsive to subsequent stimuli received via their activation receptors and NK cells that fail to engage self-MHC are considered as unlicensed (Jonsson and Yokoyama, 2009).

Structurally, inhibitory receptors belong to either the immunoglobulin like superfamily (IgSF) that includes human KIR, or to the C-type lectin-like receptor (CTLR) superfamily, which includes CD94/NKG2A and murine Ly49 molecules (Lanier, 1998). The KIR genes encode for inhibitory receptors (KIR2DL1, KIR2DL2/3, KIR2DL5, KIR3DL1 and KIR3DL2) that bind to human leukocyte antigen (HLA) class-I ligands and inhibit NK cell cytotoxicity (Lanier, 1998; Pegram et al., 2011). Similarly, the mouse has the Ly49 gene family that encodes inhibitory receptors such as Ly49A, Ly49C and Ly49I (Lanier, 1998; Pegram et al., 2011; Tomasello et al., 2000). These inhibitory receptors possess ITIMs (Immuno-receptor Tyrosine-based Inhibition Motifs) in their cytoplasmic tails. ITIMs get phosphorylated upon receptor: ligand binding, resulting in the recruitment of Src homology 2 (SH-2) domain-containing phosphatases (SHP1 and SHP2). The SHP1/2 recruitment mediates inhibition of NK cell cytotoxic activity. The NK cell inhibitory signals not only inhibit NK cell cytotoxicity but also interfere with adhesion of NK cells to target cells (Pegram et al., 2011).

1.3.2. NK cell activating receptors

The activating receptors in humans include KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1, and in mouse they include Ly49D, Ly49P and Ly49H. There are other activating receptors like, NKp46, NKp44, NKp30 and NKp80 that are found in both humans and mice (Lanier, 1998; Pegram et al., 2011; Tomasello et al., 2000). These

receptors signal through ITAM (Immuno-receptor tyrosine-like activating motifs). Unlike ITIMs, ITAMs are not present in the cytoplasmic tails of the receptors themselves; rather, the receptors associate with adaptor molecules such as CD3ζ, FcεRIγ and DAP12 (Lanier, 1998; Pegram et al., 2011). Upon ligand-receptor binding, cytoplasmic tyrosine residues get phosphorylated, which leads to recruitment of SH-2 domain-containing kinases (Syk or ZAP70). This activates a signaling cascade that results in NK cell degranulation and transcription of cytokine and chemokine genes (Lanier, 1998; Pegram et al., 2011; Tomasello et al., 2000). However, some other activating receptors, including NKG2D, use either DAP10 or DAP12 for activation. ITAMs signal through DAP12 (Lanier, 1998; Pegram et al., 2011; Tomasello et al., 2000), whereas DAP10 binds to either Grb2 or P85 and signals through phosphatidylinositol-3 kinase and other pathways (Sutherland et al., 2002; Wu et al., 1999). The subsequent signaling cascade for either DAP12 or DAP10 is not very well characterized but it is known that both pathways lead to different outcomes. DAP12 signaling results in both cytokine secretion and cytotoxicity whereas DAP10 signaling results in cytotoxicity alone (Zompi et al., 2003).

A variety of infections, including CMV in both humans and mice, can induce expression of host stress proteins such as RAE1, MULT1 and H60 in mice, and the ULBP and MIC in humans that can be recognized by the activating receptor NKG2D. The importance of this recognition and defense system is supported by the observation that both MCMV and HCMV have evolved an array of evasive genes that interfere with the surface expression of NKG2D ligands on infected cells, and manipulation of these genes can result in a virus with reduced virulence (Guma et al., 2006; Vidal and Lanier, 2006). Besides activating and inhibitory receptors, NK cells also possess co-stimulatory receptors like, NKR-P1 and

DNAM-1. These receptors help in NK cell activation along with other receptors, but alone are not sufficient to exert their function. Therefore, they provide an alternative mechanism for NK activation (Pegram et al., 2011). NK cells also express cytokine receptors and hence can respond to myriad cytokines (Biron et al., 1999). Taken together, the presence of different receptors on NK cells reveals their heterogeneous nature and also indicates that NK cells can play differential roles during an immune response.

1.4 NK cell effector functions

NK cells can employ different pathways to kill their target: direct cytolysis of the target cell, antibody-dependent cellular cytotoxicity (ADCC) and pro-inflammatory cytokine secretion. NK cell mediated cytotoxicity has been documented in different viral infections such as, LCMV (Welsh, 1978), MCMV (Orange and Biron, 1996a; Orange and Biron, 1996b; Orange et al., 1995), HSV (Ching and Lopez, 1979), influenza virus (Santoli et al., 1978) and Coxsackie virus (Godeny and Gauntt, 1986). Type I interferons such as, IFNα/β induce NK cell mediated cytotoxicity during viral infection. As mentioned earlier, during some infections, the infected cells tend to down-modulate MHC class I expression as a mechanism to evade recognition by T cells. Therefore, in the absence of MHC class I mediated inhibitory signal, signals from activating receptors lead to the induction of NK cell cytotoxic functions and cytokine secretion (Biron et al., 1999; Newman and Riley, 2007).

The NK cell-mediated cytotoxicity is driven by perforin and granzyme filled granules present inside the cell. Upon target cell recognition, these granules are released in the proximity of cells. Perforin is a pore forming protein that creates a pore in the target cells and

through these pores granzyme, a serine protease, will enter inside the cell, resulting in apoptosis by activating the caspase pathway (Smyth and Trapani, 1995). NK cells can also express surface receptors for immunoglobulin, FcγR and hence can be activated through these receptors to mediate cytotoxicity, the process commonly known as ADCC. The binding of the Fc portion of an antibody to FcγR triggers NK cells to release cytotoxic granules containing perforin and granzyme leading to target cell lysis (Perussia et al., 1989). NK cell mediated ADCC is shown to be important in limiting HIV infection (Forthal et al., 1999; Forthal et al., 2001).

In addition to cytotoxicity, NK cells also produce pro-inflammatory cytokines IFN-γ and TNF-α in response to infection. NK cell derived IFN-γ is observed during various viral infections such as MCMV (Orange and Biron, 1996b) and influenza virus (Monteiro et al., 1998). IL-12 produced by DCs alone or in combination with IL-15 induces IFN-γ production by NK cells, whereas IFNα/β stimulates cytotoxicity (Ferlazzo and Munz, 2004; Ferlazzo et al., 2004). IL-12 induces STAT4 phosphorylation and dimerization that results in IFN-γ production by NK cells (Nguyen et al., 2002). NK cell derived IFN-γ is critical in limiting early viral replication, induction of MHC class I expression on macrophages and in inducing T cell differentiation into Th1 cells (Boehm et al., 1997). IFN-γ is thought to stimulate expression of several genes that exhibit potent antiviral functions. Additionally, NK cells produce another pro-inflammatory cytokine, TNF-α that induces apoptosis of infected cells during MCMV infection (Zhou et al., 2007). Besides IFN-γ and TNF-α, NK cells also produce other cytokines such as IL-13 and GM-CSF (Cooper et al., 2001). Taken together, NK cells, as a heterogeneous population, employ various pathways to mediate pathogen clearance.

1.5 Immune responses to CMV

Both innate and adaptive immune responses are involved in providing protection against CMV. NK cells, macrophages, DCs and neutrophils are innate immune cells that mediate protection during initial stages of CMV infection before the adaptive immune response gets activated. The cytokines produced during innate phase not only interferes with viral growth and replication but also shape the adaptive immune response. T and B cells are lymphocytes of the adaptive immune response that help in viral clearance from the host and also generate a long lasting immunological memory.

1.5.1 Cytokines and chemokines

The early immune response against a virus is critical in controlling the virus replication and protecting the host from excessive immune mediated pathology. TLR3 and TLR9 are involved in early recognition of MCMV infection and activation of various genes involved in the innate and adaptive immune responses (Tabeta et al., 2004). TLR3 recruits TIR domain-containing adaptor–inducing IFN-β (TRIF). TRIF then mediates activation of IFN regulatory factor 3 (IRF3) through a series of steps, resulting in transcription of IFN-β genes (Kawai and Akira, 2006). Conversely, signaling through TLR9 results in IRAK1 mediated IRF7 phosphorylation. The phosphorylated IRF7 then dimerizes and translocates to the nucleus where it regulates the expression of type I IFNs (Kawai and Akira, 2006). Once MCMV infection is established, NK are activated by cytokines and chemokines, including type I IFN and IL-12, secreted by DCs (Garcia-Sastre and Biron, 2006; Tabeta et al., 2004). However, there are also reports suggesting that, during MCMV infection, cross talk between

pDCs and NK cells can inhibit IL-12 production, leading to suppression of IFN-γ-producing NK cells (Dalod et al., 2003).

The innate immune cells also secrete chemokines during MCMV infection that help in recruiting effector cells to the site of infection. Macrophage inflammatory protein 1α (MIP-1α), MIP-1β and RANTES are some of the inflammatory chemokines that are produced during infection. MIP-1α and β chemokines belong of the CC family, and are important for virally induced inflammation and NK cell migration into tissues (Cook et al., 1995; Salazar-Mather et al., 1998). These chemokines are produced by a variety of cell populations like macrophages, activated NK cells and T cells. The secreted chemokines bind to either CCR5 or CCR1 receptors expressed on macrophages, NK cells and DCs, and induce a signaling cascade that results in the chemotaxis of immune cells to the site of infection (Dorner et al., 2004). Taken together, some cytokines help in limiting viral replication, whereas chemokines accelerate the viral clearance by recruiting more effector cells at the site of infection.

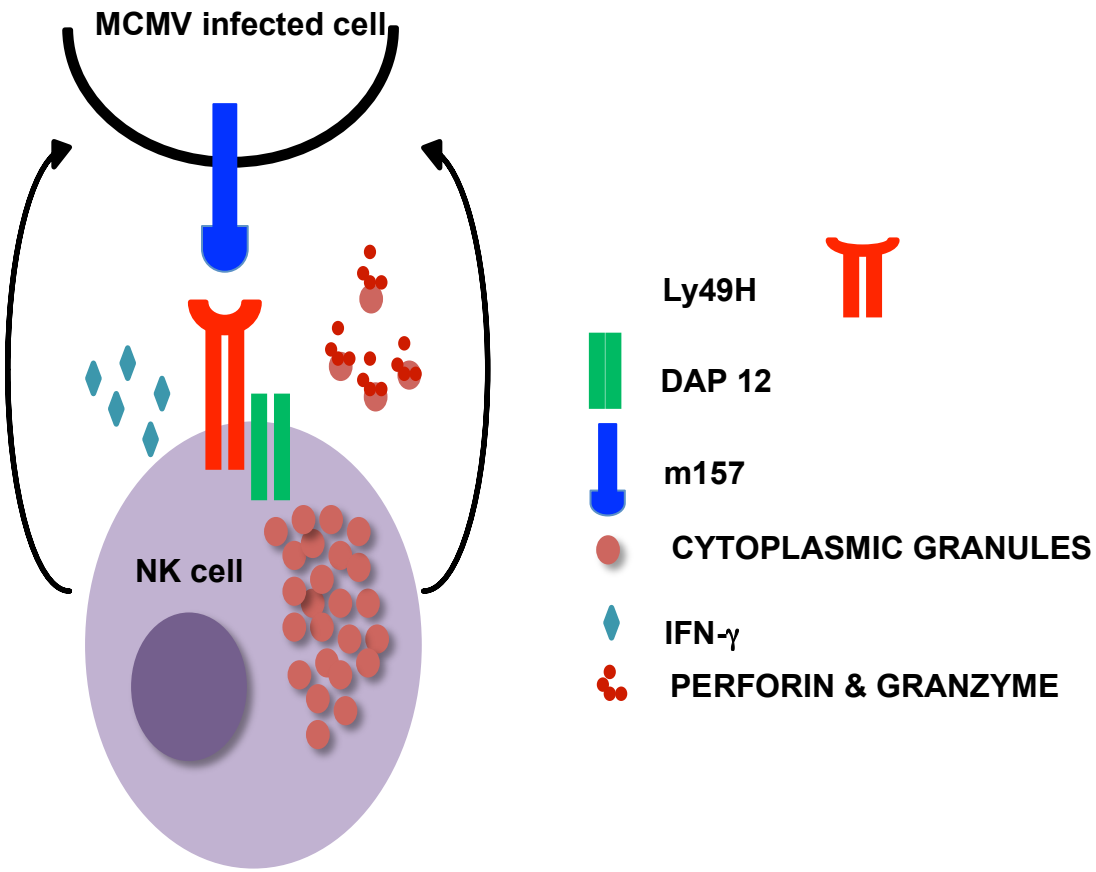
1.5.2 NK cells

NK cells make an essential contribution to mammalian innate immune defense against HCMV and MCMV. During some infections, NK cells can directly sense viral pathogens through a specific and complex set of inhibitory and activating receptors that recognize viral proteins. A clear example of direct recognition of virally infected cells by an NK cell receptor is the binding of the m157 MCMV gene product on MCMV-infected cells by the mouse Ly49H stimulatory receptor (Arase et al., 2002; Smith et al., 2002). The Ly49H receptor gene is a member of the C-type lectin-like Ly49 receptor family found in the

NK gene complex (NKC). Ly49H signaling is dependent on ITAM-containing adaptor molecules, DAP12, that get phosphorylated upon Ly49H-m157 binding, which in turn activates down stream signaling pathways for cytokine secretion and cytotoxicity (Smith et al., 1998) (**Figure 1.5)**. MCMV infection is characterized by nonspecific and specific phases of NK cell activation *in vivo.* The nonspecific NK cell activation occurs without regard to Ly49H expression during early phases of infection. Subsequently, preferential proliferation of Ly49H expressing NK cells occurs that could be inhibited by α-Ly49H treatment, indicating that Ly49H itself was specifically stimulated in MCMV infection (Dokun et al., 2001). The presence of Ly49H in C57BL/6 mice confers resistance against MCMV whereas mice strains lacking Ly49H such as BALB/c are highly susceptible to infection. In resistant mice, presence of Ly49H is sufficient to protect against MCMV whereas susceptible mice need all kind of immune response to clear the infection. (Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001; Scalzo et al., 2007).

Besides being stimulated through direct recognition of MCMV infected cells, NK cells can also be activated via activating receptors that recognize altered host MHC class I molecules induced by MCMV infection. This has been exemplified in MA/My mice, where activating receptor Ly49P recognizes H2-D^k on MCMV-infected cells but not uninfected cells; this suggests that MA/My resistance is conferred by NK cell–mediated recognition of infected cells by a mechanism involving Ly49P, H2-D^k, and an additional molecule expressed during MCMV infection (Desrosiers et al., 2005). In another study, infection of MA/My mice with a MCMV strain lacking m04 protein resulted in increased susceptibility, suggesting that Ly49P-mediated NK cell recognition of H2-D^k complex with m04 on the infected cell is necessary for resistance (Kielczewska et al., 2009).

Figure 1.5 Recognition of MCMV protein m157 by NK cell activating receptor Ly49H. The schematic of activation of NK cells following recognition of MCMV infected cell through activating receptor. Upon activation, NK cells release pro-inflammatory cytokines and cytotoxic granules that mediate target cell killing.


MCMV infected cell
NK cell
Ly49H
DAP 12
m157
CYTOPLASMIC GRANULES
IFN-γ
PERFORIN & GRANZYME

1.6 Immune regulation

The response mounted by both innate and adaptive immune cells is important in controlling viral replication and generating long-lasting memory, however, it must be tightly regulated to maintain sufficient levels of immune response for pathogen elimination while avoiding excessive immunopathology. The delicate balance between immune mediated pathology and immune regulation dictates the outcome of infection. The weak host immune response may not be able to eliminate a pathogen completely from the host body, thereby resulting in persistent infection. On the other hand, an exaggerated immune response might help to resolve the infection but at the expense of excessive inflammation inducing tissue damage. Excessive inflammation is detrimental to the host and is observed in various uncontrolled infections. Sepsis associated with rapidly disseminating pathogens such as *Toxoplasma gondii* is one such example of a harmful and damaging host immune response (Gazzinelli et al., 1996)

1.6.1 Regulatory cytokines

1.6.1.1 IL-10

IL-10 is a potent immunosuppressive cytokine that inhibits excessive host-damaging immune and Th1 responses. The regulatory role of IL-10 in immune regulation became apparent when it was found that *Il10*$^{-/-}$ mice exhibited uncommonly high inflammatory responses and consequently developed inflammatory bowel disease (IBD) characterized by colitis and dysplasia (Kuhn et al., 1993). Also, mice lacking IL-10 gene specifically in T cells developed rectal prolapse, an indicator of severe intestinal inflammation at the age of 6

months, further supporting the crucial role of IL-10 in protecting the host from excessive inflammation (Roers et al., 2004). IL-10 is a pleiotropic cytokine that functions in different ways to modulate the immune response resulting in homeostasis. It suppresses the expression of MHC and co-stimulatory molecules B7-1/B7-2 on antigen presenting cells like macrophages and DCs, and therefore can interfere with activation of an effective adaptive immune response. It also limits the production of pro-inflammatory cytokines, IFN-γ, TNF-α, IL-6, IL-12, IL-18, etc. and chemokines including RANTES, MCP-1, MCP-5, IP-10 and MIP-2, thereby protecting the host from adverse effects of excessive inflammation (Couper et al., 2008; Moore et al., 2001). During mycobacterial infections, the autocrine action of IL-10 on DCs inhibits chemokine production and prevents their migration to lymph nodes (Demangel et al., 2002). Interestingly, IL-10 can directly modulate CD4+ T cell mediated immune responses by inhibiting the production of various cytokines (Joss et al., 2000; Moore et al., 2001).

IL-10 can function in two ways. It can protect the host from excessive inflammation as exemplified in infections like Toxoplasmosis, thereby providing the means for counter-regulation to maintain immune balance (Gazzinelli et al., 1996). The regulatory role of IL-10 has been demonstarted in other infections, for example, *Mycobacterium* spp. (Murray and Young, 1999) and HSV (Suvas et al., 2004). Alternatively, it can interfere with effective T cell responses resulting in excessive viral loads and chronic infections. Excessive IL-10 can inhibit the pro-inflammatory response to some pathogens, such as lymphocytic choriomeningitis virus (LCMV) (Blackburn and Wherry, 2007), *Mycobacterium* spp. (Brooks et al., 2006) and *Leishmania* spp. (Anderson et al., 2008) to an extent that the pathogen escapes the immune response and causes fulminant and persistent infection. Recently accumulating evidence

demonstrated that blockade of IL-10 receptor (IL-10R) with neutralizing antibodies during LCMV infection resulted in rapid resolution of the persistent infection that correlated with enhanced IFNγ production by anti-viral CD8+ T cells (Brooks et al., 2006; Ejrnaes et al., 2006). These observations further support the notion that persistent viral infection in mice results in a significant up-regulation of IL-10 by APCs, leading to impaired T-cell responses. IL-10 also regulates Th2 responses by limiting the production of IL-5, IL-4 and IL-13 cytokines that can lead to severe fibrosis as exemplified in *Schistosoma mansoni* infection (Wynn, 2004). Taken together, IL-10 is an important player in regulating both innate and adaptive immune responses.

1.6.1.2 Transforming Growth factor-β (TGF-β)

TGF-β is another pleiotropic cytokine that exhibits a potent immunoregulatory role and is produced by a variety of cell populations like T cells, macrophages, B cells and NK cells (Li et al., 2006). This cytokine can act on different cell populations to regulate a variety of processes ranging from cell growth, proliferation, migration, survival and differentiation, to cellular functions including wound healing, immune responses and development (Letterio and Roberts, 1998). Owing to its regulatory role, TGF-β production during helminthic colonization of mucosa suppresses colitis. This regulation was lost when TGF-β signaling was interrupted, suggesting its role in regulating intestinal cytokine responses (Ince et al., 2009). Targeted blockade of TGF-β1 signaling in T cells demonstrates that TGF-β1 is a key negative regulator in immune homeostasis. Mice lacking TGF-β1 signaling specifically in CD4+ and CD8+ T cells developed autoimmune disease. Also, the absence of TGF-β1 signaling results in

spontaneous differentiation of most T cells into Type 1/Type 2 cytokine secreting cells (Gorelik and Flavell, 2000).

Some studies have demonstrated conversion of mouse CD4+ T cells to T_{regs} by stimulating through TCRs in the presence of TGF-β (Chen et al., 2003; Fantini et al., 2004). In addition to CD4+ T cells, TGF-β also inhibits proliferation and differentiation of CD8+ T cells to CTLs (Ahmadzadeh and Rosenberg, 2005). Cazac and Roes observed hyperresponsiveness in B cells in conditional knockout mice lacking the TGF-β receptor, indicating crucial role of TGF-β in B cell homeostasis (Cazac and Roes, 2000). TGF-β down regulates antiviral responses and also inhibits IFN-γ production by NK cells (Laouar et al., 2005). The elevated TGF-β levels in cancer patients are found to be associated with down modulation of NK cell receptor NKG2D, suggesting its role in regulating cytolytic function of NK cells (Lee et al., 2004). Furthermore, TGF-β also has a critical role in regulating leukocyte functions in various autoimmune disorders, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and insulin-dependent (type I) diabetes mellitus (Li et al., 2006). All together, TGF-β plays a dominant role in limiting excessive disease mediated pathology in the host.

1.6.2 Regulatory cells

1.6.2.1 Regulatory T cells (T_{regs})

Various immune subsets like dendritic cells and macrophages can produce IL-10, but regulatory T cells (T_{reg}) are the most well known source of this cytokine. Depending upon the site of development, T_{regs} can be divided into two categories: natural (nT_{reg}) that develop in thymus are Foxp3 positive ($CD4^+CD25^+Foxp3^+$) while adaptive/induced T_{reg} that develop in

peripheral lymphoid tissues are Foxp3 negative ($CD4^+CD25^+$Foxp3) (Shevach, 2006). nT_{regs} are activated through TCR and exert their suppression activity in antigen specific fashion. nT_{regs} suppress the activation, expansion and cytokine production by CD4+ T and CD8+ T cells (Piccirillo and Shevach, 2001; Thornton and Shevach, 1998). Besides interfering with T cell activities, nT_{regs} also suppress B cell proliferation and immunoglobulin (Ig) class switching (Lim et al., 2005). They also inhibit NK cell mediated cytotoxicity and down modulate NKG2D receptor expression during tumor growth in a TGF-β dependent manner (Ghiringhelli et al., 2005). nT_{regs} exert their suppressive function in a cell-contact-dependent manner by using membrane bound molecules, such as CTLA-4 that binds to CD80 or CD86 on the surface of APCs. This cell-to-cell contact can transduce a co-stimulatory signal to nT_{reg} resulting in suppression of the immune response (Miyara and Sakaguchi, 2007).

Inducible T_{regs}, on the other hand, are different from nT_{regs} in terms of their suppression mechanisms, cytokine profile and antigen responsiveness. They can be differentiated *in vitro* from naïve CD4+ T cells by continuing TCR stimulation in the presence of immunosuppressive drugs and they function in an IL-10 dependent manner. They can inhibit T cell proliferation to comparable levels as nT_{regs} (Vieira et al., 2004). Since naturally occurring nT_{regs} represent only a small fraction of peripheral blood mononuclear cells, studies are now largely focused on inducible T_{regs} for possible therapeutic use in autoimmunity (La Cava, 2009). Nevertheless, the activity of either of these T_{regs} must be tightly regulated because too much immune suppression can make host highly susceptible to infection, whereas too low suppression can lead to autoimmune disorders (Miyara and Sakaguchi, 2007).

1.6.2.2 Regulatory NK cells

NK cells boost immune defense via their cytolytic activity and their capacity to produce interferon-γ. NK cells can also dampen immune responses to diverse pathogens either via by direct killing of DCs (Andrews et al., 2010) or by producing regulatory cytokine, IL-10 (Lee et al., 2009; Perona-Wright et al., 2009). Remarkably, this immunosuppressive activity only occurs during systemic infections. NK cells can negatively regulate antiviral T cell responses by eliminating virus specific T cells in a contact-dependent manner as exemplified in studies with hepatitis B virus (HBV). One of the hallmarks of active HBV infection in liver is up regulation of TNF-related apoptosis-inducing ligand (TRAIL) death receptor on HBV-specific T cells, but this renders them highly susceptible to NK cell mediated deletion that expresses TRAIL. The TRAIL mediated NK cell activation results in target cell killing (Peppa et al., 2013). In addition, studies with immunocompromised perforin knockout (*Prf1*$^{-/-}$) mice also depicted NK cells as major producer of IL-10 during early phases of MCMV infection that can regulate the magnitude of CD8+ T cell response (Lee et al., 2009). IL-10 produced by NK cells is important as it is produced during the early phases of infection before T cells reach the state of full activation. Therefore NK cell-derived IL-10 may play a critical role in modulating T cell responses while protecting the host from excessive immunopathology.

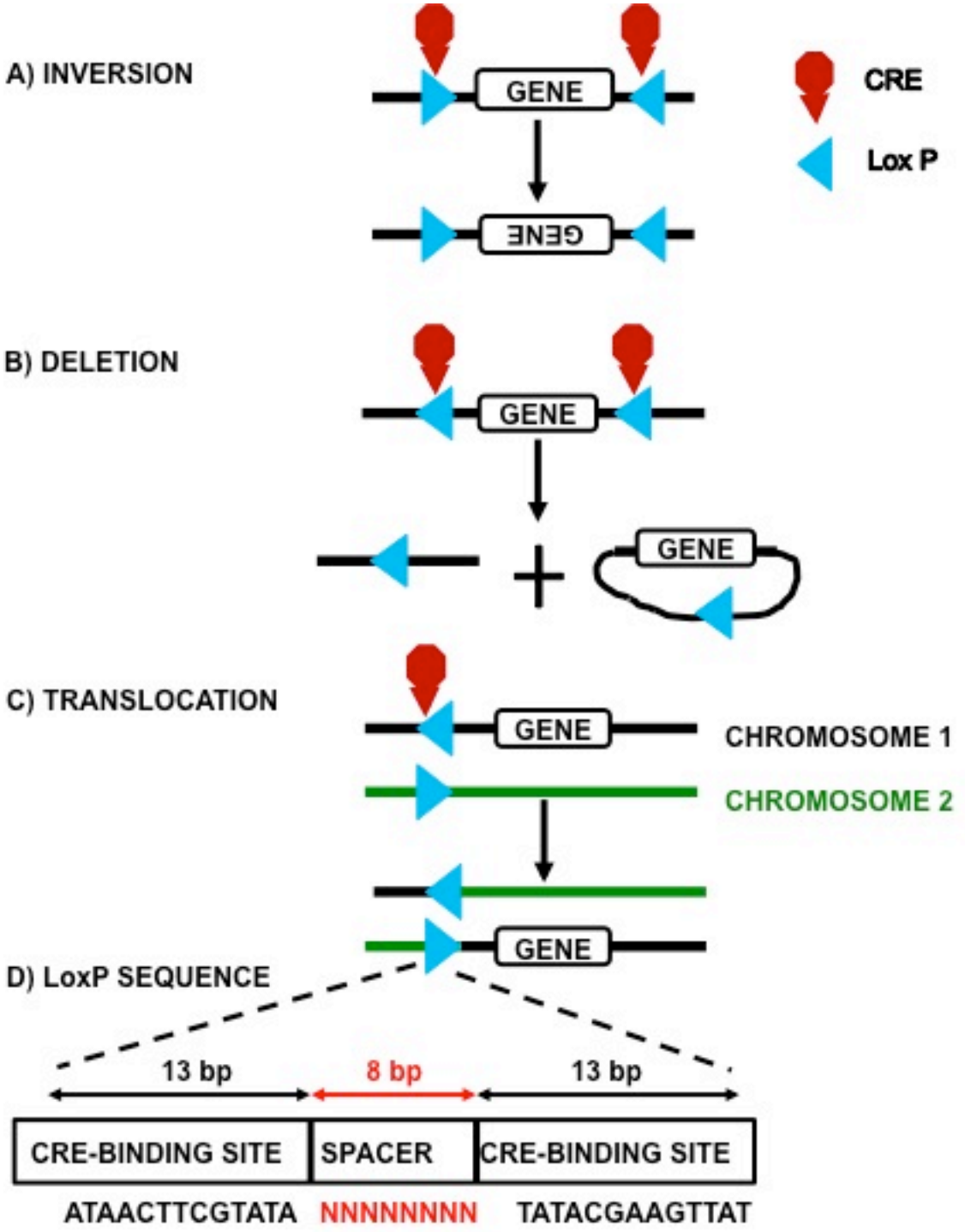
1.7 Cre-LoxP recombination

The Cre-LoxP system was first discovered in bacteriophage P1 as part of its normal viral lytic cycle. Bacteriophages use Cre-loxP recombination to circularize and facilitate replication of their genomic DNA (Sauer and Henderson, 1988; Sternberg and Hamilton,

1981). Since then, it has been developed as a powerful molecular biology tool for genome manipulation. Cre-LoxP technology requires two components: Cre recombinase, encoded by *Cre* (*C*yclization *re*combination) gene and LoxP (Locus of X-over of P1) sites. Cre recombinase is a 38 KDa protein that mediates site-specific recombination between two of its recognition sites called LoxP. LoxP is a 34 bp consensus sequences consisting of an 8 bp core spacer sequence flanked by two 13 bp palindromic sequences, as shown in **Figure 1.7D.** The asymmetric core sequence defines the orientation of the LoxP site. A single Cre recombinase molecule binds to each of the palindromic sequences of the LoxP site. The bound recombinase molecules form tetramers, that bring the two LoxP sites together, and a recombination event takes place in the spacer area between the LoxP sites (Nagy, 2000).

Depending upon the position and orientation of LoxP sites, the outcome of recombination can be: (A) if the LoxP sites are oriented in opposite direction on the same chromosome, Cre recombinase mediates inversion of flanked sequences, as depicted in **Figure 1.7A**, (B) if the LoxP sites are oriented in the same direction on same chromosome segment (cis-arrangement), Cre recombinase mediates deletion of flanked sequences, as in **Figure 1.7B**, and (C) if the LoxP sites are oriented in the same direction but on two different chromosome segments (trans-arrangement), Cre recombinase mediates translocation of flanked sequences, as in **Figure 1.7C** (Nagy, 2000). Presently, the Cre-LoxP system is most the widely used as a tool to generate conditional knockout mice. This is achieved by mating a mouse expressing *Cre* gene under the control of either an inducible or a cell-specific promoter with a mouse that carries the target gene flanked by LoxP sites on either side (also called as floxed gene) (Nagy, 2000).

Figure 1.7 Schematic depicting outcomes of Cre-LoxP recombination. Depending upon the position and orientation of LoxP sites, Cre mediated recombination can result in inversion **(A)**, deletion **(B)** and translocation **(C)**. **D)** Schematic of LoxP sequence.


A) INVERSION
GENE
GENE
CRE
Lox P
B) DELETION
GENE
GENE
C) TRANSLOCATION
GENE
CHROMOSOME 1
CHROMOSOME 2
GENE
D) LoxP SEQUENCE
13 bp
8 bp
13 bp
CRE-BINDING SITE
SPACER
CRE-BINDING SITE
ATAACTTCGTATA
NNNNNNNN
TATACGAAGTTAT

1.8 Pathogen: *Listeria monocytogenes* (LM)

LM is a gram-positive, non-spore forming, facultative aerobic bacterium that belongs to family *Corynebacteriaceae*. These bacteria can be found commonly in soil, surface water samples, sewage, slaughterhouse wastes, and human and animal feces (Farber and Peterkin, 1991). It is a food-borne pathogen and is the etiological agent for human listeriosis. The overall mortality associated with listeriosis is 30%, which can rise to 40% in case of the vulnerable individuals (Farber and Peterkin, 1991). Listeriosis results in diverse clinical manifestations, which can be attributed to the capacity of LM to cross three tight barriers within the human host. Following the ingestion of contaminated food, LM is able to cross the intestinal barrier and invade the intestinal epithelium, resulting in gastroenteritis. In addition, LM has the unique ability to cross the fetal-placental barrier in pregnant women. This can lead to infection of the fetus and septic abortion. Finally, LM is capable of crossing the blood–brain barrier and infect the meninges in the brain, resulting in potentially fatal meningitis and encephalitis (Pamer, 2004). LM is an obligate intracellular pathogen that infects macrophages and uses a potent virulence factor, LLO, to escape from the phagosome and thus gain access to the host cell cytoplasm (Mackaness, 1962). Within the cytosol, bacterial replication begins and host actin filaments (ActA) coat the surface of LM to form a polarized tail that allows for intracellular movement and intercellular spread. This cell-to-cell spread allows LM to largely avoid the extracellular milieu, thereby escaping the neutralizing effects of antibodies and the complement system (Hamon et al., 2006). Both innate and adaptive immune responses are required for successful clearance of LM infection. The importance of cytokines produced by innate immune cells in limiting LM growth has been shown by studies with Severe combined immunodeficient (SCID) mice and nude mice (Bancroft et al., 1991; Nickol and Bonventre, 1977). Macrophages internalize bacteria from the bloodstream after TLR mediated

recognition. Once inside the cells, LM secretes LLO that activates NF-κB mediated transcription of innate immune response genes. IFN-γ secretion by other innate immune cells and also by NK cells is essential for this initial defense against LM (Havell, 1989; Pamer, 2004). Furthermore, the secretion of chemokine CCL2 by macrophages recruits monocytes to the site of infection. The monocytes then get activated through their TLRs resulting in release of TNF and reactive nitric oxide (NO) that mediated target cell killing (Pamer, 2004).

Additionally, the role of CD8+ T cells is indispensable in mediating clearing intracellular pathogens and providing long-lasting memory (Ladel et al., 1994). The CD8+ T cells are activated by APCs that can present bacterial proteins in context of either MHC class I and II. Following antigen stimulation, CD8+ T cells can either employ perforin/granzyme-mediated cytolysis (White et al., 2000) or FAS mediated lysis to clear the infection (Jensen et al., 1998). Furthermore, CD8+ T cells also produce pro inflammatory cytokines IFN-γ and TNF-α during LM infection. The mouse listeriosis is a widely used model to study adaptive immune responses against pathogens. However, the low frequency of antigen specific CD4+ and CD8+ T cells makes it difficult to study dynamics of T cell responses (Jensen et al., 1998). In addition to the classical immune responses described above that are induced during LM infection, host immunoregulatory pathways also become activated to limit the magnitude and duration of immune responses and to prevent excessive immunopathology.

1.9 Statement of objectives

The main objective of my thesis project is to characterize IL-10 production, to generate *NK-Il-10-/-* mouse strain and to dissect the immunoregulatory role of NK cells during MCMV and LM infections.

The specific aims of this thesis are:

1. Demonstrate IL-10 production by NK cells during MCMV and LM infection
2. Generation and characterization of NK cell specific *Il-10* knockout mouse (*NK-Il-10-/-*) by:
    a. Confirming *Il-10* gene deletion in NK cells
    b. Confirming absence of IL-10 protein production by NK cells
    c. Confirming absence of spontaneous inflammation in *NK-Il-10-/-* mouse
3. Dissect the role of NK cell-derived IL-10 during MCMV and LM infections.

2. Materials and Methods

2.1 Mice

BALB/c mice (8 weeks old) were purchased from Charles River, Canada. The 8 week old B6.129S6-*Il10*^{tm1Flv}/J mice (commonly known as IL-10 GFP; *tiger*) were purchased from The Jackson laboratory, USA. These mice were originally generated by insertion of an internal ribosomal entry site (IRES) and green fluorescence protein (GFP) element immediately before polyadenylation site of the *Il-10* gene as previously described (Kamanaka et al., 2006). Axel Roers from Department of Dermatology, University of Cologne, Cologne, Germany provided the *Il-10*$^{flox/flox}$ mouse. Briefly, this mouse was generated by insertion of two loxP sites flanking parts of the promoter region, transcription initiation site, and the first exon of *Il-10* allele (Roers et al., 2004). The *NKp46*^{iCre} knock-in mouse was a kind gift from Dr. Eric Vivier (Centre d'Immunologie de Marseille-Luminy, Marseille, France). This knock-in mouse was generated by homologous recombination in which *iCre* (improved Cre) was inserted at the 3' end of the *Nkp46* gene (Narni-Mancinelli et al., 2011). All mice were bred and maintained in the animal facility at the University of Ottawa in agreement with guidelines and regulations of the Canadian Council on Animal care. All experimental procedures were approved by the Animal care and veterinary services of University of Ottawa. All mice were use between the ages of 7-9 weeks for experiments.

2.2 Tail DNA extraction and genotyping

Tail DNA was extracted by using a modified alkaline lysis method (Truett et al., 2000). Briefly, 600 μl of 50 mM NaOH solution was added to 2 mm tail sample in screw cap

tube. The tubes were incubated at 95^0C for 20 min with vortexing every 5 min. After 20 min incubation, the samples were neutralized by adding 50 µl of 1 M Tris (pH 7.0) and vortexed for 30 s. The 2 µl aliquots of extracted DNA were used for PCR reactions. The primers oIMR8292, oIMR8625 and oIMR8626 were used to detect transgene IL-10 GFP in IL-10 GFP reporter mice. For *NK-Il-10-/-* genotyping, primers NCR1-Cre-F2, NCR1-Cre-KI-R and NCR1-Cre-WT-R were used to detect presence of *Cre* gene. The primers IL-10-Floxed-F and IL-10-Floxed-R1 were used to detect floxed *Il-10* gene. For *NK-Il-10-/-* mice characterization, SV151-F and SV151-R primers were used as control. The IL-10-Deleted-F2 and IL-10-Floxed-R1 primers were used to detect deleted *Il-10* gene in *NK-Il-10-/-* mice. The sequences for the primers are shown in the table below.

Table 2.1: List of primer sequences

NAME OF THE PRIMER	PRIMER SEQUENCE
oIMR8292	5'- CCAAAAGACGGCAATATGGT-3'
oIMR8625	5'- GTGTGTATTGAGTCTGCTGGAC-3'
oIMR8626	5'- GTGTGGCCAGCCTTAGAATAG-3'
NCR1-Cre-F2	5'-GCAAAGCATTCTAAAGGACACTG-3'
NCR1-Cre-KI-R	5'-CCCTAGGAATGCTCGTCAAG-3'
NCR1-Cre-WT-R	5'- TTCCCGGCAACATAAAATAAA-3'
IL-10 Floxed-F	5'- CCAGCATAGAGAGCTTGCATTACA-3'
IL-10 Floxed-R1	5'- TCCTCTTGGGATCCAGTTGT-3'
IL-10-Deleted-F2	5'-GCTGCTTCTCCTGCTGAGTT-3'
SV151-F	5'-GTGCTACCACTGAAAACCATTG-3'
SV151-R	5'-CTGTCTCTTGAGTCACCTGCAC-3'

2.3 Pathogen

2.3.1 MCMV

The Smith strain MCMV (ATCC® VR-1399™) was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The MCMV stocks were prepared in our laboratory. Four weeks old BALB/c mice were infected with 5×10^3 plaque-forming units (PFUs) of MCMV. After 21 days of infection, mice were sacrificed and salivary glands were harvested and pooled in 50 ml of Media 199 (Gibco, cat # 11043). The harvested salivary glands were washed once with Media 199 and then added in 10 ml of Media 199. The mixture was then homogenized at speed of 15 for 10 s or until fully homogenized by using a Polytron homogenizer (Model: PT 1600E). The homogenized samples were centrifuged at 2500 rpm for 15 min at 4°C. The supernatant was then aliquot in 1.5ml volume and stored as master stock in liquid nitrogen. Three days later, working stock was prepared from master stock by aliquoting into 30 µl volume and stored at -80°C. For virus titration, one vial from working stock was thawed, serially diluted 10-fold in chilled 2% DMEM (DMEM containing 2% FBS). PFUs of these dilutions were determined in triplicates by standard plaque assay.

2.3.2 *Listeria monocytogenes* (LM)

A listeriolysin-positive, streptomycin-resistant strain of LM (10403S) was a kind gift from Dr. Subash Sad lab (University of Ottawa, Ottawa, Ontario). The bacteria were aliquoted, frozen in 20% glycerol and stored at -80°C. Colony forming units (CFUs) were determined by performing serial dilutions in 0.9% NaCl, which were spread on brain heart infusion (BHI) agar plates (Difco laboratories).

2.4 Mouse Embryonic Fibroblast (MEF) cell preparation

Embryos were obtained from BALB/c mice at 14-15 days of gestation and placed in sterile 80 mm petridish containing phosphate buffer saline (PBS). With the help of forceps, organs such as, liver, heart and blood were removed. The remaining tissues were minced with the help of two pairs of forceps to pieces of about 3 mm diameter. The minced embryos were washed three times with PBS and trypsinized with 0.05% Trysin-EDTA (Wisent Inc. cat # 325-042-CL) at 4°C overnight. Next day, trypsin was pipetted out carefully while working on ice, the embryos containing residual trypsin was incubated at 37°C for 15 min. Warm 10% DMEM (10% FBS, 1X antibiotics, 25 mM HEPES) was added and a single cell suspension was prepared by using a cell dissociation sieve tissue grinder kit (Sigma CD1-1KT). Samples were centrifuged at 500 g for 10 min and the cells were resuspended in 2 ml of 10% DMEM per embryo. The cell concentration was determined and cells were seeded at 5×10^5/ml on DMEM on 14 cm tissue culture dishes. When fully confluent, cells were recovered by washing first with warm PBS, detaching with 5 ml of 0.05% Trypsin-EDTA and suspending in 10% DMEM. The cells were pooled together and centrifuged at 500 g for 10 min, resuspended in sterile ice-chilled DMEM containing 20% FBS and 10% DMSO and aliquot at a concentration of 2×10^6 cells/ml. The aliquots were stored in liquid nitrogen.

2.5 MCMV plaque assay

Frozen MEF cells were thawed and rinsed with 10% DMEM (10% FBS, 1X antibiotics, 25 mM HEPES). The cells were cultured in 150 mm tissue culture dish (Thermo Scientific, cat # D6JA55I104) until they reached confluency. One day before plaque assay, cells were seeded at a concentration of 1.6×10^5 cells/ml in 10% DMEM. After incubation at 37°C for 24 h in 5% CO_2 incubator, the monolayer was washed with 2% DMEM (DMEM

containing 2% FBS) and infected with 200 µl of serial dilutions of organ homogenates (1/2 for spleen, 1/5 for liver, 1/20 and 1/100 for salivary glands). To prepare the organ homogenates, small pieces of the organ were cut, weighed and placed in screw cap tube containing 2% DMEM and two SS grinding 5 mm balls (Fisher Scientific, cat # FSSP9729110). The tubes were then placed inside the tube holder of MagNA Lyser (Roche) and homogenized at 7000 rpm for 15 s for salivary glands and the homogenate was then diluted appropriately. The virus was allowed to adsorb to cells for 1 h at 37°C in 5% CO_2 incubator and then the plate was overlaid with DMEM containing low melting (LM) agar. The media containing LM agar was prepared by dissolving 12.5 ml 2% LM in DMEM and 37.5 ml DMEM containing 13.5% FBS. Plaques were allowed to develop for three days. After three days, cells were fixed with 10% formalin (Protocol, cat # 245-684) for 10 min and stained with 0.1% crystal violet (in 70% ethanol).

2.6 Colony Assay

Spleens of infected mice were homogenized in 0.9% NaCl by using frosted ends of microscope slides (*Fisher*brand, cat # 12-552). The homogenate was diluted appropriately and 100 µl of 10-fold serial dilutions was plated on BHI agar plates (Difco laboratories). The plates were incubated at 37 °C for 24 h. The colonies are counted to determine the bacterial burden.

2.7 Single cell lymphocyte suspension preparation

The spleen was harvested and transferred to 6-well plate containing 5 ml RPMI-1640 media/well (HyClone, cat # SH30027.01). The single cell suspension was prepared by

grinding spleen on 70 μm nylon cell strainer (*Fisher*brand, cat # 22363548) using the end of a 3 ml syringe plunger. The cells were lysed with Ammonium-chloride-potassium (ACK) buffer, suspended in RPMI-1640 media and centrifuged at 1200 rpm for 10 min at 4°C. For liver cell isolation, the harvested liver was transferred to a well in a 6-well plate containing 5 ml of RPMI-1640 media/well. The liver was transferred onto the surface of 70 μm nylon cell strainer and ground by using the end of a 3 ml syringe plunger until all cells had passed through the strainer. The cell suspension was transferred to a 15 ml tube and centrifuged at 1200 rpm for 10 min at 4°C. The cells were washed thrice with RPMI-1640 media. After the third wash, the cells were suspended in 8 ml room temperature (RT) 42% Percoll (GE Healthcare, cat # 17-0891-01) and carefully overlaid onto 3 ml RT 73% Percoll. Centrifuged at 2400-2500 rpm for 20-25 min at RT with no breaks. After centrifugation, the top fat layer was suctioned off using a Pasteur pipette and the lymphocyte ring was collected into a new tube. The lymphocytes were suspended in RPMI-1640 media and centrifuged at 1200 rpm for 10 min at 4°C.

For lymphocyte preparation from salivary glands, the harvested salivary gland was placed in 35 mm × 10 mm dish (Corning, cat # 430165) containing PBS. Lymph nodes were removed from the salivary gland by using forceps and scissors and the salivary gland was then transferred into a new 35 mm × 10 mm dish containing 2 ml of enzyme cocktail (Collagenase type XI 125 U/ml (50 μl of 12,500 U/ml stock, C7657 Sigma), 60 U/ml of Hyaluronidase type I-s (5 μl of 60,000 U/ml stock, H3506 Sigma), 60 U/ml of DNase I (20 μl of 6,000 U/ml stock, D4527 Sigma) in 5 ml of HBSS w/o $CaCl_2$, $MgCl_2$, and $MgSO_4$ (Gibco, cat # 14170-112). The salivary gland was then finely chopped with scissors and incubated at 37°C for 30 min with continuous shaking in Shake 'n' Bake hybridization oven

(Model # 136400 from Boekel). After every 15 min, the sample was taken out and pipetted vigorously. After 30 min, the sample was transferred to 70 μm nylon cell strainer placed in 6-well plate and grinded completely with the end of a 3 ml syringe plunger. The cells were suspended in staining buffer (PBS containing 2% FBS) and centrifuged at 1250 rpm for 10 min at 20°C. The pellet was resuspended in 3.5 ml of staining buffer and carefully overlaid onto 3.5 ml Lympholyte-M (Cedarlane, cat # CL5035) by placing Pasteur pipette to the bottom of tube. Centrifuge at 1000-1500g for 20 min at RT. After centrifugation, the lymphocyte layer from interphase was collected to new tube, resuspended in staining buffer and washed once. Cells were counted and subsequently staining.

2.8 Antibodies and Flow cytometry

For characterization of *NK-Il-10-/-* mice at genomic level, total splenocytes were labeled with α-TCR-APC (clone H57-597), α-NK1.1-PE (clone PK136) and α-CD19-APC-Cy7 (clone 1D3) and total hepatic leukocytes (to obtain NKT cells) were labeled with α-TCR-β-APC (clone H57-597) and α-NK1.1-PE (clone PK136). The labeled cells were then flow sorted by using MoFlo XDP-sorter from Beckman Coulter (Stem Core laboratories, OHRI, Ottawa) to obtain T cells, B cells, NK cells and NKT cells. For functional characterization of *NK-Il-10-/-* mice, NK cells were enriched from freshly isolated total splenocytes by negative magnetic separation using NK cell isolation kit II (cat # 130-096-892) following the manufacturer's protocol (MACS®; Miltenyi Biotec) The enriched NK cells were stimulated *ex-vivo* with 10 μg/ml each of purified mouse IgG1-κ isotype control (BD Pharmingen), purified α-Ly49H (clone 3D10) from eBiosciences, functional grade purified α-NK1.1 (clone PK136) from eBiosciences and cytokines IL-2 (1000 U/ml) + IL-12

(50 ηg/ml) for 1 h at 37°C. To estimate the degranulation, stimulated cells were then incubated with α-CD107a-FITC (clone eBio1D4B) from eBiosciences along with 5 μg/ml of Brefeldin A (BFA)(cat # B7651-5MG) from Sigma life sciences for 4 h at 37°C. After 4 h incubation, cells were used for intracellular staining. Briefly, cells were fixed (BD Cytofix/Cytoperm™, cat # 51-2090KZ) and permeabilized (BD Perm/Wash™, cat # 51-2091KZ) and labeled with α-IFN-γ-APC (clone XMG1.2).

The cell content in different organs such as, spleen, liver and salivary gland was determined by surface staining. Single cell suspensions (1×10^6 cells) was incubated at 4°C for 15 min with α-CD16/32 (clone 2.4G2, from Bioexpress) to reduce non-specific binding. Cells were labeled with various combinations of directly conjugated monoclonal antibodies (0.25 μl of each antibody/million cells) and incubated at 4°C for 25 min. α-CD49b-PE (clone DX5), α-NK1.1-PE (clone PK136), α-Ly49H-FITC (clone 3D10) (all three antibodies were purchased from eBiosciences) and α-NK1.1-APC (clone PK136) from BD Pharmingen were used to determine NK cells number and percentage. α-TCR-β-FITC (clone H57-597) or α-TCR-APC (clone H57-597) or α-TCR-APC-Cy7 (clone H57-597) from eBiosciences were used to determine T cell. α-CD8a-450 (clone 53-6.7) from eBiosciences and α-CD4-500 (clone RM4-5) from BD Horizon™ were used to determine CD8+ T cell and CD4+ T cell number and percentage respectively.

Intracellular staining was used to measure cytokine, IFN-γ level. Briefly, the cells were incubated with 5 μg/ml of BFA from Sigma life sciences for 4 h at 37°C and stained for surface markers. The cells were fixed (BD Cytofix/Cytoperm™, cat # 51-2090KZ) and permeabilized (BD Perm/Wash™, cat # 51-2091KZ) and labeled with α-IFN-γ-APC (clone XMG1.2) from eBiosciences. Cells were suspended in staining buffer (1X PBS, 2% FCS and

0.09 % sodium azide), acquired and analyzed using FACS Cyan ADP & Kaluza software v2 (Beckman Coulter).

2.9 Cytometric Bead Assay (CBA) for cytokine quantification

The production of cytokine, IL-10 in conditioned media (CM) (supernatant) was measured using mouse CBA kit (BD Biosciences, cat # 558267). Samples were prepared according to manufacture's instructions, acquired on FACS Cyan ADP (Beckman Coulter) and analyzed using the BD FCAP Array Software (BD Biosciences). Cytokine production is presented as pg/10^6 cells for conditioned media.

2.10 Histology

For functional characterization of *NK-Il-10-/-* mice, the colon samples were harvested from six months or older mice. The samples were flushed with PBS by using syringe and needle and immersed in 4% paraformaldehyde (PFA) for fixing with slight shaking. Fixed samples were sent to Department of pathology and laboratory medicine at University of Ottawa, Ottawa where the tissues were embedded in paraffin and hematoxylin and eosin (H&E) stained. Briefly, the fixed samples were précised, embedded and cut in pieces of 4-micron size and then stained with H&E stains. The nuclei of the cells stain blue and the cytoplasm of the cells stain pink. Slide pictures were taken at magnifications of 20.

2.11 Statistical Analysis

Significance of results was determined by two-tailed unpaired student *t* tests (*$*p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$*) and graphed using Graph Pad Prism 5 software.

3. RESULTS

3.1 Identification of IL-10 producing cells during microbial infections

It has been demonstrated previously that NK cells play an important immunoregulatory role by producing IL-10 and therefore prevent CD8+ T cell-mediated immunopathology during uncontrolled MCMV infection in immunodeficient *Prf-/-* mice (Lee et al., 2009). Hence based on previous studies with immunodeficient mice, I decided to investigate the immunoregulatory role of NK cells during immunocompetent conditions using C57BL/6 IL-10 GFP reporter mice. The use of this IL-10 GFP reporter mouse is advantageous over IL-10 intracellular staining to measure IL-10 expression because signals obtained by staining are often weak and it is hard to interpret which positively stained cells provided the secreted IL-10. Furthermore, since GFP is inserted in the 3' untranslated end of the *Il-10* gene locus in the reporter mouse, GFP expression can be directly correlated to *Il-10* gene expression (Kamanaka et al., 2006). Such mice have been previously used to study the role of intestinal T cell derived IL-10 in regulating gut inflammation (Kamanaka et al., 2006), B cell derived IL-10 during autoimmunity (Scapini et al., 2011) and IL-10 mediated regulation of liver inflammation during acute MCMV infection (Gaddi et al., 2012). These previous studies have demonstrated that combining GFP analysis with multicolor staining enables easy identification of IL-10 producing population. Therefore, I sought to investigate IL-10 production by NK cells in immunocompetent conditions using IL-10 GFP reporter mice. Despite the insertion, IL-10 production is not hampered in this mouse; nonetheless to avoid any discrepancies associated with interfering effects of the inserted GFP reporter gene on endogenous IL-10 expression, I used only heterozygous IL-10 GFP knock-in mice.

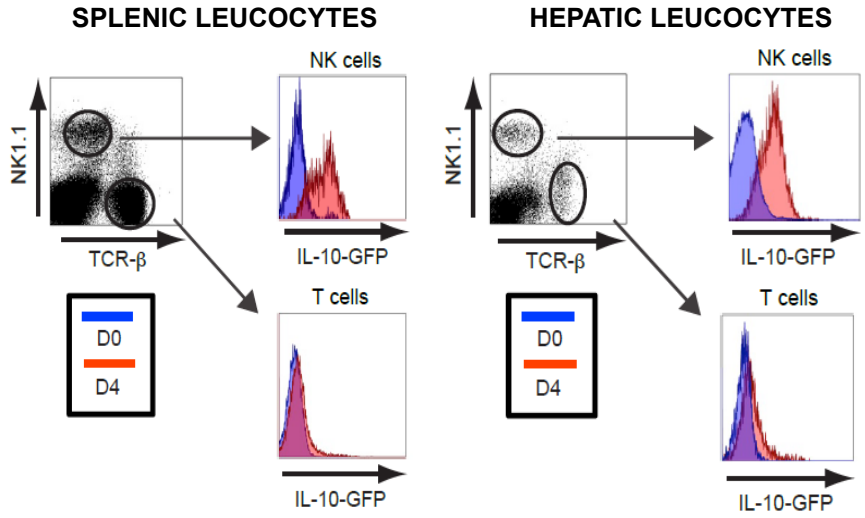
3.1.1 Kinetics of IL-10 production during sustained MCMV infection

The mice were infected with 1.2×10^4 PFU of MCMV and IL-10 GFP expression was observed by flow cytometry on different days: D2, D4, D6, D8, D10 and D12 post infection. Briefly, three mice per group were infected intraperitoneally with above-mentioned dose of MCMV on different days whereas tissues were harvested on one day. On the day of experiment, all mice were sacrificed; spleen and liver were isolated and used for preparing single cell suspension of lymphocytes. Flow cytometric analysis showed that NK cells as detected by NK1.1 expression were predominantly IL-10 GFP expressing on D4 of MCMV infection in both spleen and liver. The overlay plot comparing IL-10 GFP expression on D0 and D4 was prepared from gated NK and T cells. In splenic leucocytes, NK cells predominantly expressed IL-10 GFP on D4 post infection **(Figure 3.1.1A)**. Similarly in hepatic lymphocytes, IL-10 GFP expression was mainly observed in NK cells on D4 post infection **(Figure 3.1.1A)**. On the other hand, almost negligible IL-10 GFP expression was observed in T cells from both the organs.

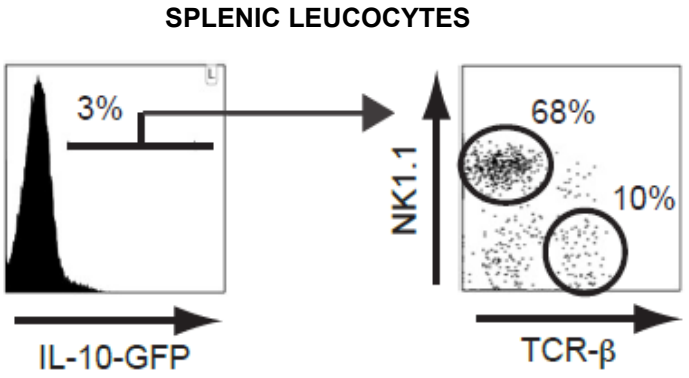
To further confirm that NK cell contribute maximally to total IL-10 GFP expression on D4, I performed back gating analysis to further confirm percentage of IL-10 expressing NK cells among total splenic leucocytes. Based on my back gating data, I confirmed that indeed NK cells are major producer of IL-10 on D4 post infection. Among total IL-10 GFP expressing cells, approximately 68% were NK cells and only 10% were T cells **(Figure 3.1.1B)**. The complete kinetics of IL-10 production during MCMV infection is obtained from flow cytometric analysis of splenocytes. The IL-10 GFP expression by NK cells starts as early as D3 with a peak observed on D4 of infection. After D4, the IL-10 GFP expression slowly starts to diminish in NK cells with returning to basal level on D8 of infection. On D4 approximately 80% of NK cells expressed IL-10 GFP **(Figure 3.1.1C)**. Among T cells, IL-

Figure 3.1.1 NK cells are major producers of IL-10 during initial stages of MCMV infection. A) The overlay plots depicting IL-10 GFP expression in NK cells and T cells are prepared from total splenic and hepatic leukocytes on D4 post MCMV infection (n=3, 1.2×10^4 PFU/mouse). ***B)*** Back gating analysis representing IL-10 producing NK cells and T cells among total IL-10 GFP expressing cells. The percentages represent the proportion of IL-10 GFP expressing cells. **C)** The graph represents the complete kinetics of IL-10 GFP expression during MCMV infection. The proportion of IL-10 GFP expressing NK cells and CD4+ T cells are obtained from total splenic leucocytes. Each point depicts mean ± S.D. of three mice. The NK cells are gated as NK1.1+TCR-β- and T cells are gated as TCR-β+NK1.1-.

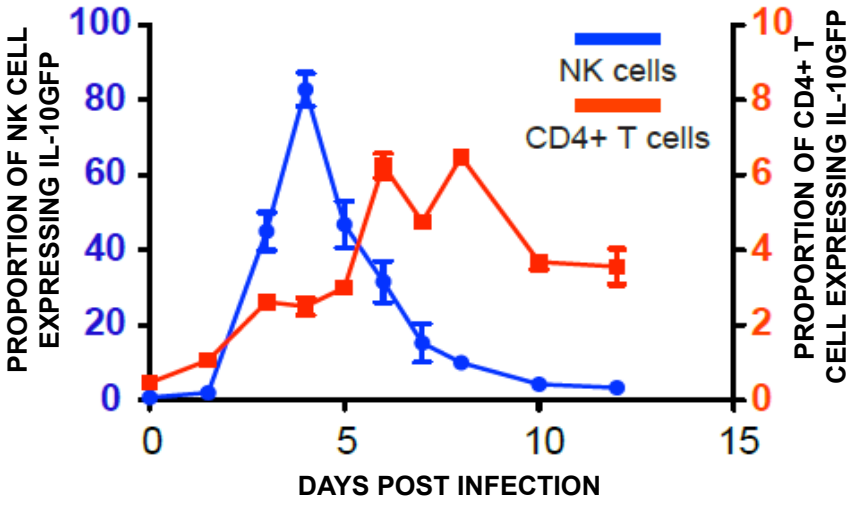
A


SPLENIC LEUCOCYTES
HEPATIC LEUCOCYTES
NK cells
NK1.1
TCR-β
IL-10-GFP
T cells
D0
D4

B


SPLENIC LEUCOCYTES
3%
IL-10-GFP
68%
10%
NK1.1
TCR-β

C


PROPORTION OF NK CELL EXPRESSING IL-10GFP
PROPORTION OF CD4+ T CELL EXPRESSING IL-10GFP
NK cells
CD4+ T cells
DAYS POST INFECTION

10 GFP expressing cells were mostly CD4+ T cells. Although GFP expression by CD4+ T cells overlapped with NK cells, only a small proportion of CD4+ T cells expressed IL-10 as compared to NK cells during initial stages of infection. The IL-10 GFP expression by CD4+ T cells gradually increased as infection progressed with the peak observed on D8 and then it started decreasing **(Figure 3.1.1C)**. Approximately 6% of CD4+ T cells were IL-10 GFP positive on D8 of infection. Taken together, my data strongly demonstrate that NK cells are the major producer of the regulatory cytokine IL-10 during early stages of MCMV infection in immunocompetent mice. Furthermore, NK cells and CD4+ T cells participate in a division of labor for maintaining constant level of IL-10 during sustained MCMV infection.

3.1.2 Kinetics of IL-10 production during chronic LM infection

In addition to viral infections, previous studies have reported IL-10 production by NK cells during acute bacterial and parasitic infections such as systemic *L. monocytogenes* (Perona-Wright et al., 2009) and *Yersinia pestis* infections (Vivier and Ugolini, 2009). The mouse model of LM infection is most widely used to elucidate different aspects of immune response and therefore, I set out to examine regulatory role of NK cells derived IL-10 during LM infection. Although I used age and sex matched littermates for various time points in my previous MCMV study, it is hard to control variations among different groups. Therefore to avoid any variations, I infected a single group of (n=6) male IL-10 GFP reporter mice with 4 $\times$ 10^4 CFUs of LM bacteria and bled them every other day post infection. A kinetic study of IL-10 GFP expression by NK cells in total blood lymphocytes showed that IL-10 expression started immediately after infection with maximum expression observed on D4. The IL-10 production by NK cells eventually decreased as infection progressed. Unlike the case with MCMV infection, only approximately 30% were IL-10 GFP expressing among total NK

cells whereas similar to MCMV, CD4+ T cells expressed IL-10 GFP in later phases of infection, with the peak observed at D10 of infection **(Figure 3.1.2A)**.

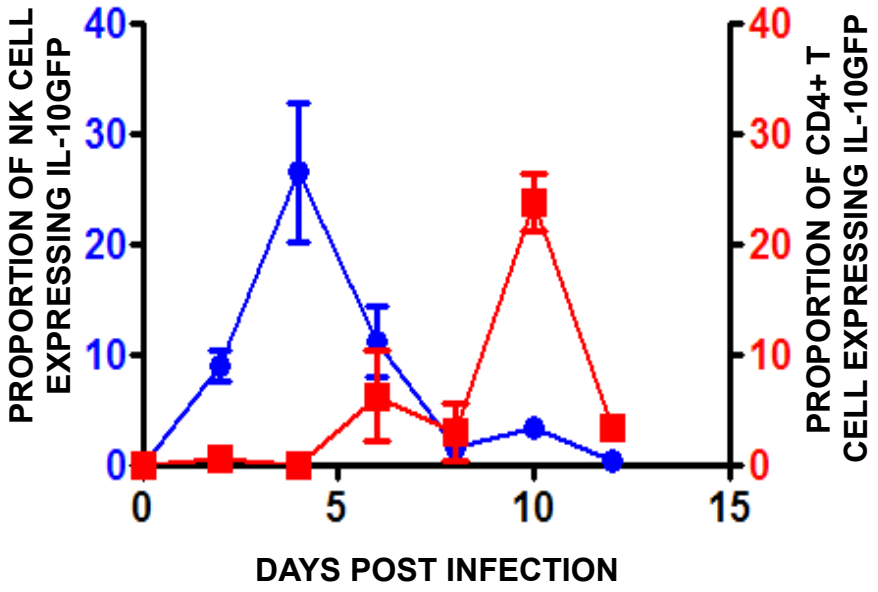
Approximately 20% CD4+ T cells were IL-10 GFP expressing on D10 post LM infection. I performed back gating analysis on total blood leucocytes to further confirm that NK cell contributes maximum to total IL-10 GFP expression on D4. Similar to my MCMV data, I confirmed that among total IL-10 GFP expressing cells, approximately 85% were NK cells and only 7% were T cells **(Figure 3.1.2B)**. Interestingly, I observed same trend of division of labor among NK cells and CD4+ T cells in both the infections but the peak in IL-10 expression by CD4+ T cells differed slightly between the infections. Taken together, my data demonstrated that NK cells are major producer of regulatory cytokine IL-10 during LM and MCMV infections, suggesting that the IL-10 production by NK cells occurs in a variety of infections.

3.2 Generation of NK cell specific knockout (*NK-Il-10-/-*) mice

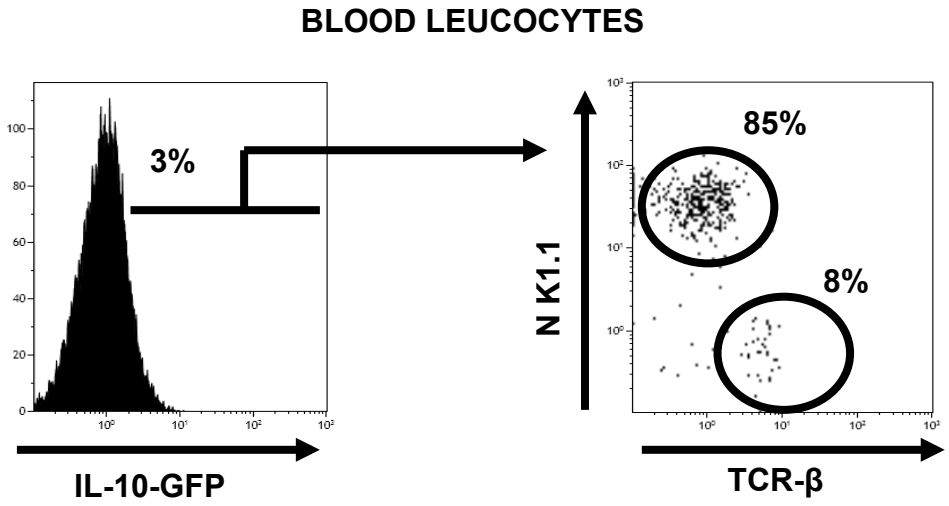
Having confirmed that indeed NK cells are major producers of IL-10 during early stages of MCMV and LM infection, I decided to elucidate the function of NK cell derived IL-10 during these infections. The use of neutralizing antibodies against the protein or its receptor has been a commonly used approach to study its function. Since different cell populations can produce IL-10 at different time points during infection and different cell subsets possess IL-10 receptor, so it is hard to control IL-10 kinetics by using IL-10 neutralizing antibody. Complete IL-10 knockout (*Il-10-/-*) mice have also been used in previous studies to elucidate the role of IL-10 globally, but severe spontaneous inflammation observed in this mouse excludes its use for infection studies. Therefore to avoid these

Figure 3.1.2 NK cells are major producers of IL-10 during initial stages of LM infection. A) The complete kinetics of IL-10 GFP expressing NK cells and CD4+ T cells are obtained from total blood lymphocytes during LM infection. The graph represents proportion of IL-10 expressing lymphocytes at indicated time points (4×10^4 CFUs/mouse, mean ± S.D., n=6). **B)** Back gating analysis representing IL-10 producing NK cells and T cells among total IL-10 GFP expressing cells on D4 of LM infection. The percentages represent the proportion of IL-10 GFP expressing cells. The NK cells are gated as NK1.1+TCR-β- and T cells are gated as TCR-β+NK1.1-.

A


PROPORTION OF NK CELL EXPRESSING IL-10GFP
PROPORTION OF CD4+ T CELL EXPRESSING IL-10GFP
40
30
20
10
0
0
5
10
15
DAYS POST INFECTION

B


BLOOD LEUCOCYTES
3%
IL-10-GFP
N K1.1
85%
8%
TCR-β

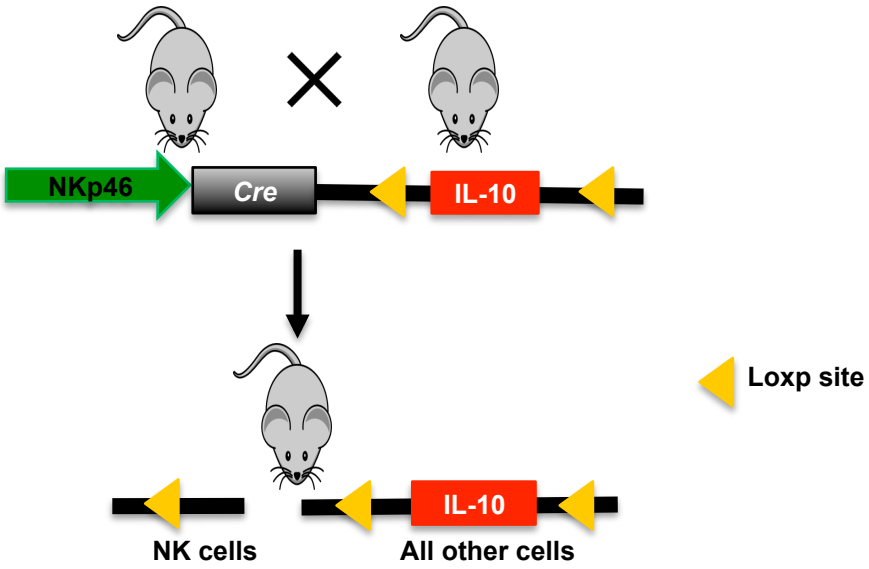
disadvantages, I generated a conditional knockout mouse that lacked *Il-10* gene specifically in NK cells. I used a well-known molecular biology technique, Cre-Loxp recombination, to generate NK cell specific IL-10 knockout (*NK-Il-10-/-*) mouse. This mouse was generated by crossing IL-10 floxed mouse (*IL-10* $^{flox/flox}$) with *NKp46*^{iCre} knockin mouse **(Figure 3.2.1A)**.

In *NKp46*^{iCre} knockin mouse, *Cre* recombinase is expressed under the control of the NKp46 promoter, which is exclusively found in NK cells (Gazit et al., 2006; Reynders et al., 2011; Walzer et al., 2007). The faithful expression of the *Cre* gene in NK cells has been confirmed previously (Narni-Mancinelli et al., 2011). I tested NKp46 expression in wildtype mice, mice heterozygous for *NKp46*^{iCre} and mice homozygous for *NKp46*^{iCre} by flow cytometry. I observed reduced NKp46 expression on NK cells of mice homozygous for the *NKp46*^{iCre} knockin allele. Similar unusual phenotype of NK cells has been reported previously (Narni-Mancinelli et al., 2012). The NKp46 expression in wildtype and heterozygous *NKp46*^{iCre} mice was comparable **(Figure 3.2.1B)**. Therefore following the advice of the group who originally generated the *NKp46*^{iCre} mouse and based on my observation, I decided to use heterozygous *NKp46*^{iCre} mouse for my experimental studies. The partner mouse, *IL-10* $^{flox/flox}$ has been used previously to investigate the effect of T cell (Roers et al., 2004) and T_{reg} (Rubtsov et al., 2008) specific IL-10 ablation on immune response. The *NK-Il-10-/-* mice were obtained at Mendelian frequency. These mice developed normally and were fertile.

Since it is ideal to use littermates for infection studies, I used the breeding scheme as depicted in **Figure 3.2.2A** to obtain the littermates. Briefly I set up the breeding pair where both the parents are homozygous for *IL-10*$^{flox/flox}$ gene (*IL-10flox/flox*$^{t/t}$), but they differ in

Figure 3.2.1 Expression of NKp46 is intact in heterozygous *NKp46^{icre}* mouse. A) Schematic cartoon showing generation of *NK-Il-10-/-* mice using *NKp46^{iCre}* and *Il-10 $^{flox/flox}$* mice. **B)** NKp46 expression on NK1.1+ TCR-β- NK cells is measured by flow cytometry. NKp46 expression on NK cells is rescued in mice heterozygous for *NKp46^{iCre}* allele whereas NKp46 expression is severely down modulated on NK cells of mice homozygous for *NKp46^{iCre}* allele.

A


NKp46
Cre
IL-10
Loxp site
IL-10
NK cells
All other cells

B

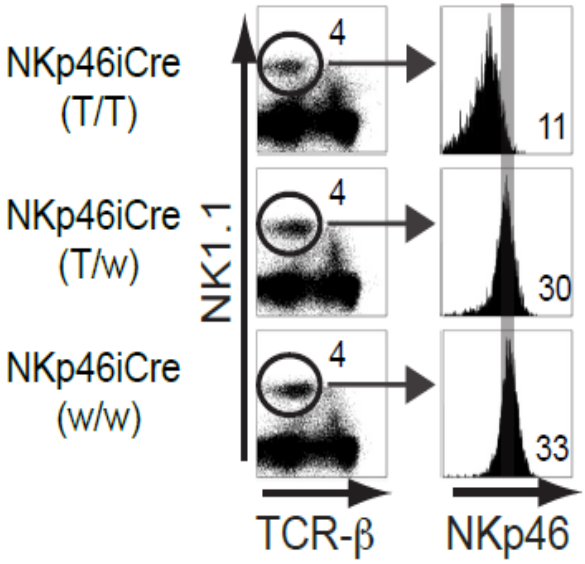
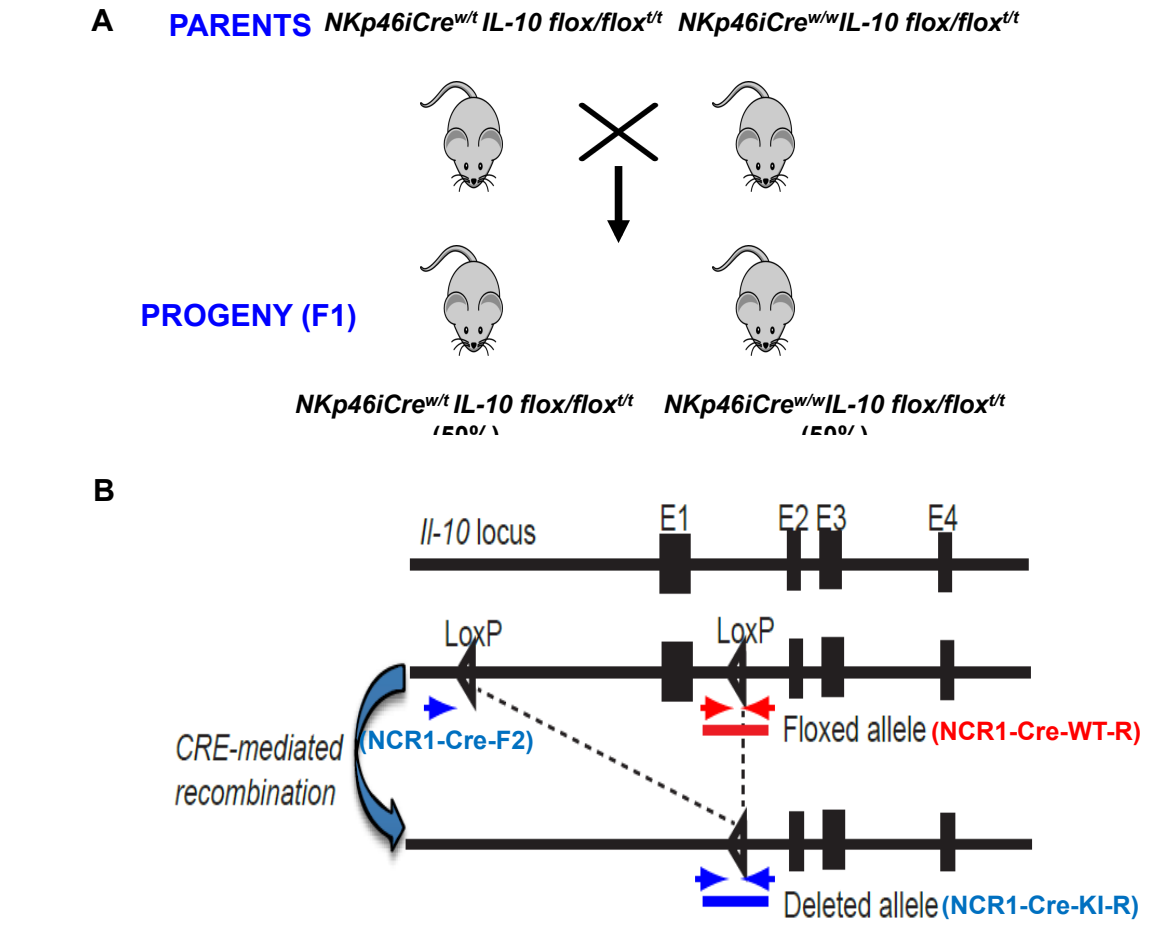

NKp46iCre
(T/T)
NKp46iCre
(T/w)
NKp46iCre
(w/w)
NK1.1
4
4
4
11
30
33
TCR-β
NKp46

Figure 3.2.2 NK cell specific IL-10 knock out (*NK-Il-10-/-*) mouse is generated by using Cre-LoxP technology. A) Schematic showing breeding scheme to generate littermates. **B)** Schematic diagram representing location of gene specific primer used to determine *Cre* mediated recombination in *NK-Il-10-/-* mice**. C)** Figure depicting PCR analysis to identify *Cre* expressing mice. The upper band corresponds to wild type (***w***) gene (no *Cre*) whereas lower band corresponds to transgenic (***t***) gene (*Cre* expressing). Well # 1to #4 represent data from *NKp46iCre*$^{w/w}$ mice whereas well #5 from *NKp46iCre*$^{w/t}$ mouse **D)** Figure depicting PCR data for identification of mice expressing floxed gene. The upper band corresponds to transgenic (***t***) (floxed gene) whereas lower band corresponds to wild type (***w***) gene. Well #1 and #2 represent *IL-10flox/flox*$^{w/t}$ mice and well #3 represents *IL-10flox/flox*$^{t/t}$ mice. PCR is performed using gene specific primer and 100 bp ladders are used to determine the size of PCR product. The arrow corresponds to respective band position.


A
PARENTS NKp46iCre$^{w/t}$ IL-10 flox/flox$^{t/t}$ NKp46iCre$^{w/w}$IL-10 flox/flox$^{t/t}$
PROGENY (F1)
NKp46iCre$^{w/t}$ IL-10 flox/flox$^{t/t}$
NKp46iCre$^{w/w}$IL-10 flox/flox$^{t/t}$
B
Il-10 locus
E1
E2 E3
E4
LoxP
LoxP
CRE-mediated recombination
(NCR1-Cre-F2)
Floxed allele (NCR1-Cre-WT-R)
Deleted allele (NCR1-Cre-KI-R)
C
Cre gene identification
#1 #2 #3 #4 #5
LADDER (Cre$^{w/w}$) (Cre$^{w/t}$)
426 427 428 429 430
300bp
247bp
w
t
D
IL-10 fl/fl gene identification
#1 #2 #3
LADDER (IL-10 fl/fl$^{w/t}$) (IL-10 fl/fl$^{t/t}$)
270bp
250bp
t
w

NKp46iCre gene. One parent is wild type *NKp46*^{iCre} mouse (*NKp46iCre*$^{w/w}$) and other heterozygous *NKp46*^{iCre} mouse (*NKp46iCre*$^{w/t}$). Upon mating, there is a 50% chance of getting progeny with genotype *NKp46iCre*$^{w/w}$ × *IL-10flox/flox*$^{t/t}$ and 50% *NKp46iCre*$^{w/t}$ × *IL-10flox/flox*$^{t/t}$. For genotyping, I used primers NCR1-Cre-F2, NCR1-Cre-KI-R and NCR1-Cre-WT-R to detect the *Cre* gene and primers IL-10-Floxed-F and IL-10-Floxed-R1 to detect the floxed *Il-10* gene. The primer sequences have been described previously in **Table 2.1** and the location of primer sets to identify IL-10 floxed and IL-10 deleted allele is shown in **Figure 3.2.2B**.

To identify the IL-10 floxed allele, I designed a forward primer complementary to sequences before the second flox site and the reverse primer complementary to sequences behind it **(Figure 3.2.2B)**. The *Cre* mediated recombination will result in deletion of the first exon of *Il-10* gene along with first flox site bringing the forward primer that was designed to detect the deleted allele close to IL-10 floxed reverse primer **(Figure 3.2.2B)**. PCR amplification for detecting *Cre* gene resulted in two bands of varying sizes. The upper band corresponds to amplification product of wild type (*w*) gene and its approximate size is 300 bp whereas the lower band represents amplification product of transgenic (*t*) gene of approximate size of 250 bp **(Figure 3.2.2C)**. Similarly, identification of floxed gene resulted in PCR products of varying sizes with upper band corresponding to transgenic gene (floxed *Il-10*) with approximate size of 270 bp and lower band corresponding to wild type gene and its size is 250 bp **(Figure 3.2.2D).** All together, I was able to design appropriate primer sets and obtain optimal PCR conditions for genotyping *NK-Il-10-/-* mice.

3.3 Characterization of NK cell specific knockout (*NK-Il-10-/-*) mice

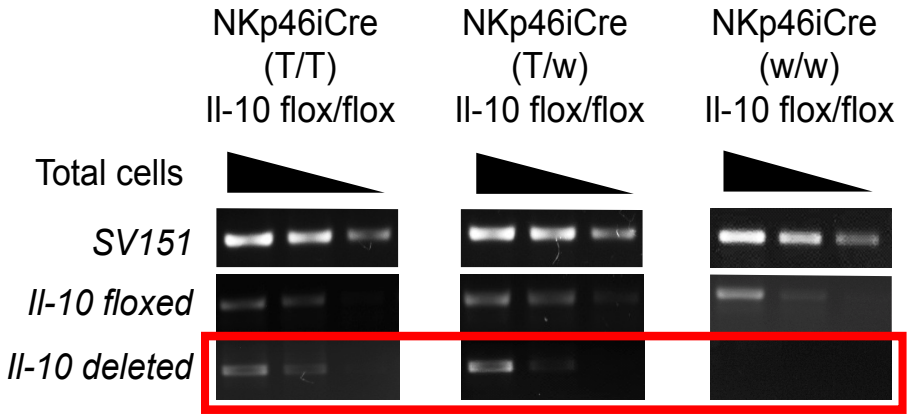
3.3.1 Evaluation of *Cre* mediated recombination in *NK-Il-10-/-* mice

To determine whether the *Cre* mediated recombination at the genomic level is dependent on gene dosage, I extracted DNA from total cells obtained from a mouse with no *Cre*, a mouse heterozygous for *Cre* and a mouse homozygous for *Cre*. I performed semi-quantitative PCR with 5-fold serial dilutions of genomic DNA using gene-specific primers sets: SV151, IL-10 floxed and IL-10 deleted. The sequences of these primer sets are described earlier in **Table 2.1**. The SV151 is polymorphic marker derived from *Ly49h* genomic sequence (Lee et al., 2001) and is also unlinked to *Il-10* gene locus, thereby used as control input DNA. The location of the primer sets to identify IL-10 floxed and IL-10 deleted allele is shown in **Figure 3.2.2B**. The genomic PCR results revealed that the deleted allele is present only in mice expressing *Cre* gene **(Figure 3.3.1A)**. This confirms that recombination occurred faithfully only in mice expressing *Cre* gene.

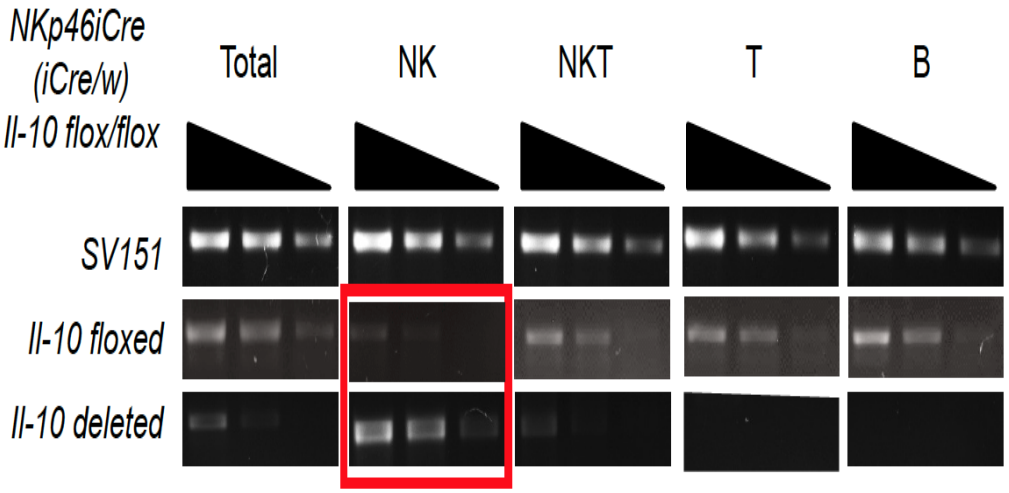
To determine whether *Cre* mediated recombination occurs specifically in NK cells, I flow sorted T cells, B cells, NK cells from spleen and NKT cells from liver of heterozygous *NK-Il-10-/-* mouse. I extracted DNA from all these different lymphocyte populations and performed semi-quantitative PCR using gene-specific primers sets: SV151, IL-10 floxed and IL-10 deleted. Notably, the IL-10 deleted allele was predominantly observed in NK cells **(Figure 3.3.1B)**. Furthermore, IL-10 floxed allele was absent in NK cells but present in all other lymphocyte populations indicating that *Cre* mediated recombination had occurred exclusively in NK cells but not in other cell populations such as T cells, B cells and NKT cells **(Figure 3.3.1B)**. Due to the presence of a small proportion of NK cells among total cells, a faint band of IL-10 deleted allele was observed in total cells too but none in T cells, B

Figure 3.3.1 *Cre* mediated recombination occurs predominantly in NK cells A) PCR analysis of genomic DNA from *Il-10*$^{flox/flox}$ mice, heterozygous *Nkp46*^{iCre} mice and homozygous *Nkp46*^{iCre} for the presence of IL-10 floxed allele and IL-10 deleted allele by using gene specific primers. The IL-10 deleted allele is observed only in mice expressing *Cre* gene. **B)** PCR analysis of genomic DNA from heterozygous *NKp46*^{iCre} mice for the presence of IL-10 floxed or deleted alleles. Genomic DNA was isolated from the indicated flow-sorted subsets and semi quantitative PCR was performed with 5-fold serial dilutions of genomic DNA using gene-specific primers. IL-10 deletion is predominant in NK cells. SV151 marker, unlinked to *Il-10* locus, is used as a control input DNA.

A


NKp46iCre
(T/T)
Il-10 flox/flox
NKp46iCre
(T/w)
Il-10 flox/flox
NKp46iCre
(w/w)
Il-10 flox/flox
Total cells
SV151
Il-10 floxed
Il-10 deleted

B

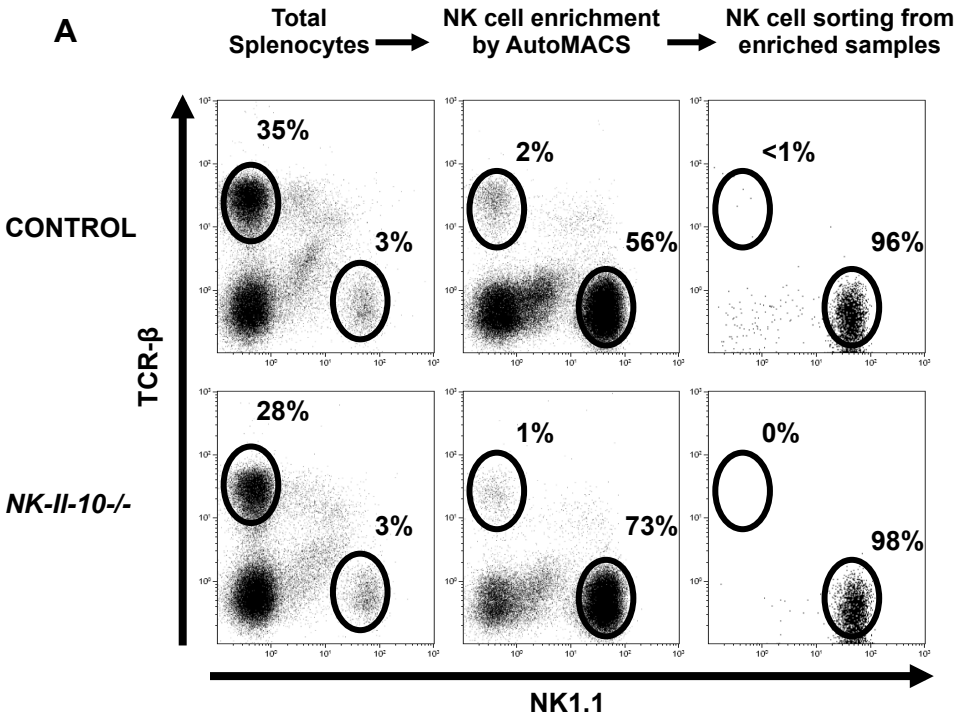

NKp46iCre
(iCre/w)
Il-10 flox/flox
Total
NK
NKT
T
B
SV151
Il-10 floxed
Il-10 deleted

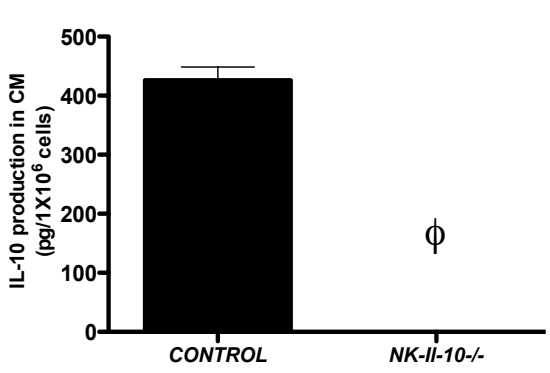
cells and NKT cells **(Figure 3.3.1B)**. Taken together, PCR analysis confirmed that *Cre* mediated recombination resulted in *Il-10* gene deletion specifically in NK cells whereas it was intact in all other lymphocyte populations.

3.3.2 Verification of absence of IL-10 in *NK-Il-10-/-* mice at protein level

IL-2 is a pleiotropic cytokine and is required for activation and proliferation of various lymphocytes, like T cells and NK cells. Resting NK cells express IL-2Rβγ and are therefore capable of responding to IL-2 without any prior activation. IL-12 is another pro-inflammatory cytokine known to globally promote NK and T cell activation and cytotoxicity (Trinchieri, 2003). IL-2 and IL-12 mediated NK cell activation result in enhanced NK cell cytotoxicity and IFN-γ production (Kobayashi et al., 1989; Yu et al., 2000). It has also been reported previously that the synergistic action of IL-2/IL-12 induces IL-10 production by NK cells (Chakir et al., 2001; Wolk et al., 2002). Therefore, I decided to confirm loss of IL-10 function in naïve NK cells following *ex vivo* IL-2/IL-12 stimulation. I enriched NK cells from total splenocytes of mice expressing no *Cre* (Control from now on) and *NK-Il-10-/-* mice. I obtained approximately 56% NK cells in control and 73% NK cells in *NK-Il-10-/-* mice **(Figure 3.3.2A)**. To obtain highly pure NK cells, I further sorted the enriched NK cells based on NK1.1 by flow cytometry. I obtained NK cells with purity of >95% **(Figure 3.3.2A)**. I stimulated the highly pure NK cells *ex vivo* in the presence of IL-2/IL-12 at 37^0C. After 18 h of incubation, I collected the CM without disturbing the underlying cells. I performed CBA on collected CM and observed approximately 450 pg IL-10/ million NK cells in CM obtained from control mouse but no IL-10 protein was detected in CM obtained from *NK-Il-10-/-* mouse. **(Figure 3.3.2B)**. This data further confirmed the loss of IL-10

Figure 3.3.2 Absence of IL-10 protein observed in CM from naïve *NK-Il-10-/-* mice. A) Highly pure NK cells from naive control and *NK-Il-10-/-* mice were obtained by NK cell enrichment followed by sorting. **B)** Highly pure NK cells from naive control and *NK-Il-10-/-* mice were stimulated with IL-2/IL-12 for 18 hours. The IL-10 production in CM is analyzed by CBA. No IL-10 is detected in CM from *NK-Il-10-/-* mice. (mean ± S.D, n=3).


A
Total Splenocytes
NK cell enrichment by AutoMACS
NK cell sorting from enriched samples
CONTROL
NK-Il-10-/-
TCR-β
NK1.1
35%
3%
2%
56%
<1%
96%
28%
3%
1%
73%
0%
98%


B
IL-10 production in CM (pg/1X10^6 cells)
500
400
300
200
100
0
ϕ
CONTROL
NK-Il-10-/-

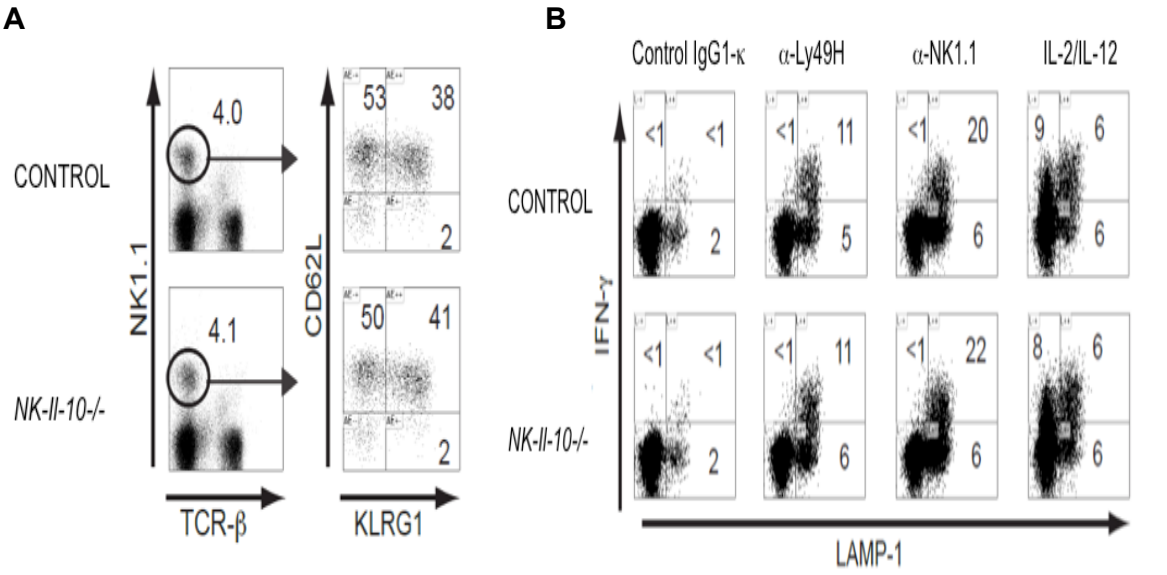
production by NK cells in *NK-Il-10-/-* mouse whereas IL-10 production was intact in control mouse.

3.3.3 Evaluation of spontaneous inflammation in *NK-Il-10-/-* mice

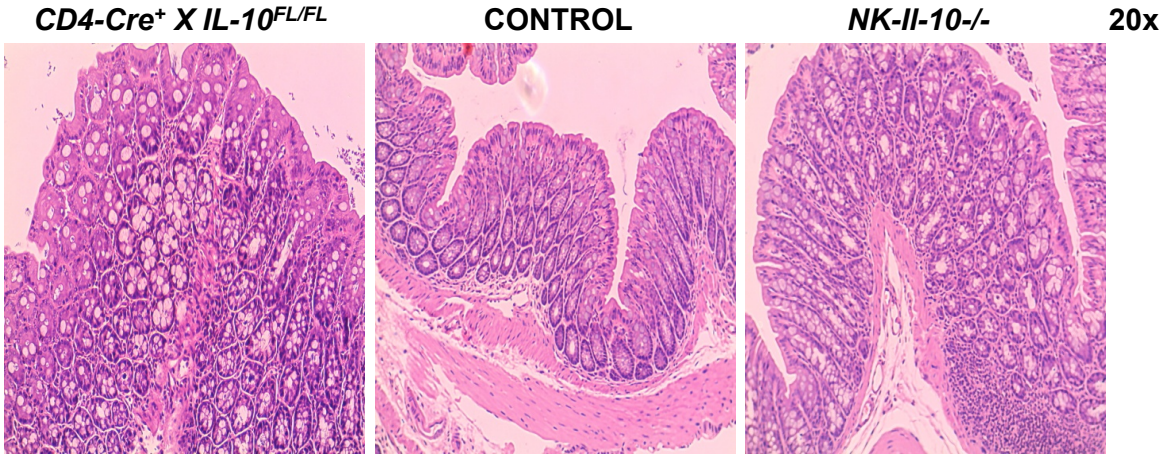
3.3.3.1 NK cell phenotype

Strikingly, most *Il10*-/- mice on the B6 background develop rectal prolapse and diarrhea; indicators of severe spontaneous intestinal inflammation at 6 months of age (Kuhn et al., 1993). Under these conditions of spontaneous inflammation, like other lymphocytes, NK cells tend to redistribute in different organs and tissues and exert their effector functions at the site of infection. Various adhesion molecules are responsible in guiding NK cells to the site of infection and CD62L is one such adhesion molecule expressed by NK cells. Some studies have reported enhanced frequency and number of CD62L expressing NK cells in various organs following infection (Morris and Ley, 2004). Similarly expression of killer cell lectin- like receptor G1 (KLRG1) on NK cells is up regulated following infection (Robbins et al., 2002). Therefore I set out to compare the phenotype of NK cells in six-month-old naïve *NK-Il-10-/-* and control mice to determine signs of spontaneous inflammation in terms of abrogated expression of activation markers. I isolated total splenic leucocytes from naïve control and *NK-Il-10-/-* mice. Flow cytometric analysis revealed comparable expression of activation markers such as CD62L, and KLRG1 on NK cells of *NK-Il-10-/-* and control mice **(Figure 3.3.3A)**. In addition, the numbers of lymphoid and myeloid subsets was comparable in both the mice.

Figure 3.3.3 No signs of spontaneous inflammation observed in *NK-Il-10-/-* mice. A) The frequency and activation status of NK1.1+ TCR-β- NK cells is determined by flow cytometry. The NK cells from control and *NK-Il-10-/-* mice showed comparable expression of activation markers CD62L and KLRG1. **B)** The enriched NK cells from naïve control and *NK-Il-10-/-* mice are stimulated with control IgG1-κ, α-Ly49H, α-NK1.1, IL-2/IL-12 and media alone for 1 hr at 37°C. The IFN-γ production and degranulation level (LAMP-1 expression) is comparable in both groups. The numbers represent percentages of cells in respective gate. **C)** Colon histology of more than 6 months old *CD4-Cre*$^{+}$ *Il-10*$^{fl/fl}$ (positive control) control (negative control) and NK-*Il-10-/-* mice (n=5) is performed. Tissues are fixed in 10% formaldehyde and paraffin embedded. Histological sections are shown at a magnification of 20x. No sign of spontaneous inflammation is observed in colon of NK-*Il-10-/-* mice whereas *CD4-Cre*$^{+}$ *Il-10*$^{fl/fl}$ exhibit hyperplasia and disorganized crypt indicative of severe inflammation.


A
CONTROL
NK-Il-10-/-
NK1.1
TCR-β
4.0
4.1
CD62L
KLRG1
53
38
2
50
41
2
B
Control IgG1-κ
α-Ly49H
α-NK1.1
IL-2/IL-12
CONTROL
NK-Il-10-/-
IFN-γ
LAMP-1
<1
<1
2
<1
11
5
<1
20
6
9
6
6
<1
<1
2
<1
11
6
<1
22
6
8
6
6

C


CD4-Cre+ X IL-10FL/FL
CONTROL
NK-Il-10-/-
20x

As mentioned previously, stimulation of NK cells by IL-2/IL-12 enhances their cytotoxicity and IFN-γ secretion as well as IL-10 secretion (Kobayashi et al., 1989; Trinchieri, 2003; Yu et al., 2000). Besides cytokine-mediated activation, NK cells can also be activated through a variety of activating receptors. Therefore, to test cytotoxic activity and pro-inflammatory cytokine production, I enriched NK cells from total splenocytes of naive control and *NK-Il-10-/-* mice and subjected them to various surface bound stimulations such as, IgG1-κ isotype control, α-Ly49H, α-NK 1.1 and IL-2/IL-12. Following stimulation, I performed intracellular staining to analyze the degranulation (in terms of LAMP-1 expression) and IFN-γ production by NK cells. The gated NK cells from control and *NK-Il-10-/-* mice showed comparable levels of IFN-γ production and degranulation level after various plate bound stimulations **(Figure 3.3.3B)** suggesting that NK cells from control and *NK-Il-10-/-* mice are phenotypically and functionally similar in naïve state.

3.3.3.2 Colon histology of *NK-Il-10-/-* mice

The regulatory role of IL-10 in limiting gut inflammation has been well documented. Most of the *Il-10-/-* mice on B6 background develop chronic enterocolitis characterized by excessive hyperplasia of mucosal epithelium; indicators of severe spontaneous inflammation at six month of age (Kuhn et al., 1993). One of the previous studies with *CD4-Cre*$^{+}$*Il-10*$^{fl/fl}$ mice showed spontaneous onset of IBD as mice aged suggesting crucial role of T cell derived IL-10 in protecting mice from excessive inflammation. The most prominent histological changes have been observed in colon of *CD4-Cre*$^{+}$*Il-10*$^{fl/fl}$ previously (Roers et al., 2004). Although T_{reg} cells have been known to play a central role in suppressing immune inflammation, it is possible that additional IL-10 sources are required to restrain spontaneous

inflammation. The mouse showing signs of spontaneous inflammation cannot be a good model for any infection studies. As NK cells from *NK-Il-10-/-* mouse do not produce IL-10, lack of this early regulatory function may render this mouse highly susceptible to spontaneous inflammation. Since the colon is most affected part during gut inflammation as exemplified in earlier studies, I decided to look at the colon histology in *NK-Il-10-/-* mouse at age six months or more and compared it with mice lacking IL-10 in T cells (*CD4-Cre*$^{+}$ *IL-10*$^{fl/fl}$). I isolated the colon from six months old *CD4-Cre*$^{+}$*Il-10*$^{fl/fl}$, control and *NK-Il-10-/-* mice and fixed in 4% PFA after thorough washing. H&E staining of colon from control and *NK-Il-10-/-* mice showed normal colon anatomy characterized by highly organized crypt structure and surface epithelium. I did not observe signs of spontaneous inflammation in *NK-Il-10-/-* mice **(Figure 3.3.3C)** as opposed to *CD4-Cre*$^{+}$*Il-10*$^{fl/fl}$ that exhibited massive hyperplasia and disorganized crypt structure indicative of spontaneous inflammation **(Figure 3.3.3C)**. This pattern of spontaneous inflammation in colon of *CD4-Cre*$^{+}$*Il-10*$^{fl/fl}$ mice was similar to what was observed previously (Roers et al., 2004). Taken together, for the first time, I have successfully generated NK cell specific *Il-10-/-* mouse that is free of spontaneous inflammation and hence is an ideal tool to elucidate the immunoregulatory role of NK cell derived IL-10 during microbial infection.

3.4 Regulatory role of NK cells during MCMV infection

To date, NK cells have been known for their critical role during acute phases of infection and production of pro-inflammatory cytokines like, IFN-γ and TNF-α. The direct anti-viral role of NK cell in containing MCMV infected cells is well known (Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001). Besides MCMV, the antiviral functions of NK

cells can also be observed in other viral infections for example, LCMV (Welsh, 1978), adenoviruses (Zhu et al., 2010), HSV-1 (Ching and Lopez, 1979), EBV, etc. (Merino et al., 1986). However, there are fewer reports about their regulatory role during immune response. Some previous studies have reported IL-10 production by NK cells during infections (discussed earlier in introduction).

3.4.1 Activation status of CD8+ T cells and NK cells after MCMV infection

In my previous results, I confirmed that NK cells are major producers of IL-10 during acute phases of MCMV infection with a peak observed on D4, which is a critical time point for T cell activation and therefore I decided to analyze effect of absence of NK cell derived IL-10 on T cell activation status during MCMV infection. One of the earlier studies had indicated that systemic but not acute infection is required to induce IL-10 production by NK cells during microbial infections (Perona-Wright et al., 2009). In addition, my data with low MCMV dose didn't depict difference in activation status of T cells as measured by expression of CD43 and CD25 markers among control and *NK-Il-10-/-* mice, indicating rapid clearance of infection in both groups. Therefore, to obtain sustained MCMV infection, I decided to infect control and *NK-Il-10-/-* mice with a high dose of MCMV, 5×10^4 PFU/mouse. To analyze the effect of NK cell derived IL-10 on T cell activation, I tested the activation status of CD4+ and CD8+ T cells and NK cells with regard to expression of activation markers CD43 and CD25 (IL-2R) by flow cytometry following MCMV infection. I observed comparable expression of CD43 and CD25 on all three-cell subsets in control and *NK-Il-10-/-* naïve mice. Notably, on D4 of MCMV infection, I observed higher expression of CD43 and CD25 on CD8+ T cells of *NK-Il-10-/-* mice as compared to control mice **(Figure 3.4.1)**. I observed slightly heightened expression of these markers on NK cells from *NK-Il-*

10-/- mice suggesting possible autocrine role of NK cell derived IL-10 in regulating NK cell activity. I didn't observe any difference in activation status of CD4+ T cells among control and *NK-Il-10-/-* mice **(Figure 3.4.1)**. I have repeated this experiment again keeping all the conditions same as I used in first experiment but unfortunately I was not able to reproduce the similar results. In my opinion, it will be worth to pursue this study to examine the immunoregulatory role of NK cells in modulating T cell response at the time of its activation.

3.4.2 Increased susceptibility of *NK-Il-10-/-* mice during MCMV infection

The production of IL-10 by NK cells during initial stages of MCMV infection has been described previously in section 3.1.1. To dissect the immunoregulatory role of NK cells, I infected the control and *NK-Il-10-/-* mice with 5×10^4 PFU of MCMV. The loss in body weight is one of the characteristic features of infection-induced disease caused by MCMV (Oakley et al., 2008), hence I measured body weight for 16 days p.i. However, both groups of mice showed comparable body weight changes over the course of infection with maximum drop in body weight observed around D4-D5 **(Figure 3.4.2A)** and recovery starting after D5, with animals reaching to their pre infection weight on D15-D16. This unexpected lack of difference in body weight loss may be due to the presence of residual IL-10 by other cell populations such as CD4+ T cells and T_{regs}. Those mice that recovered their pre-infection weight on D16 of infection were analyzed for pathology. The spleen from D16 control mice appeared almost similar to D0 control mice. However, I observed severe gross necrosis, discoloration of spleen and they were smaller in size in *NK-Il-10-/-* mice as compared to control mice **(Figure 3.4.2B)**. I enumerated total splenic lymphocytes from D16

Figure 3.4.1 NK cell derived IL-10 modulates T cell response in spleen during MCMV infection. The expression of activation markers CD43 and CD25 on different cell subsets in spleen of control and *NK-Il-10-/-* mice is determined by flow cytometry. Enhanced activation of CD8+ T cells and NK cells in *NK-Il-10-/-* mice is observed on D4 after MCMV infection (n=2 for control and n=4 for *NK-Il-10-/-* mice, 5×10^4 PFU/mouse).

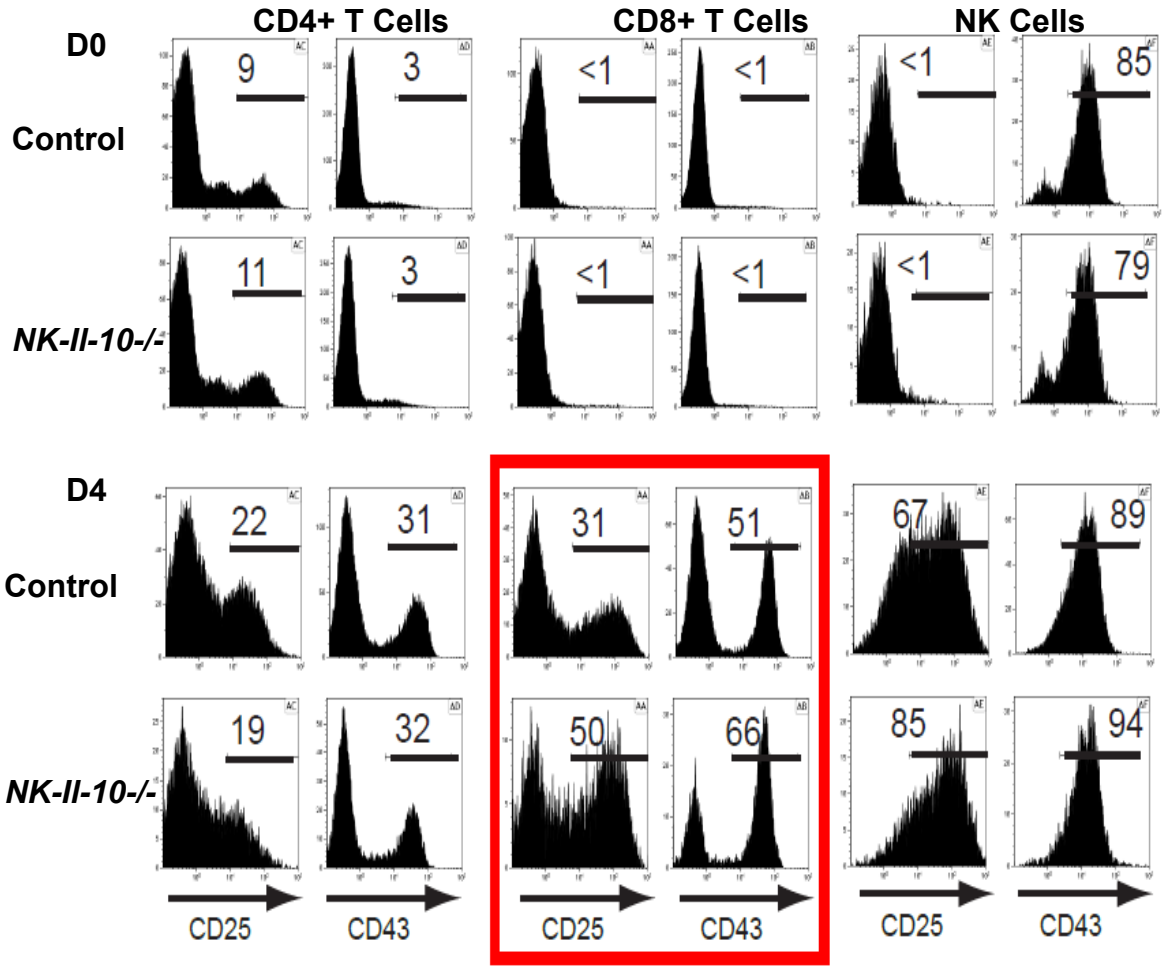
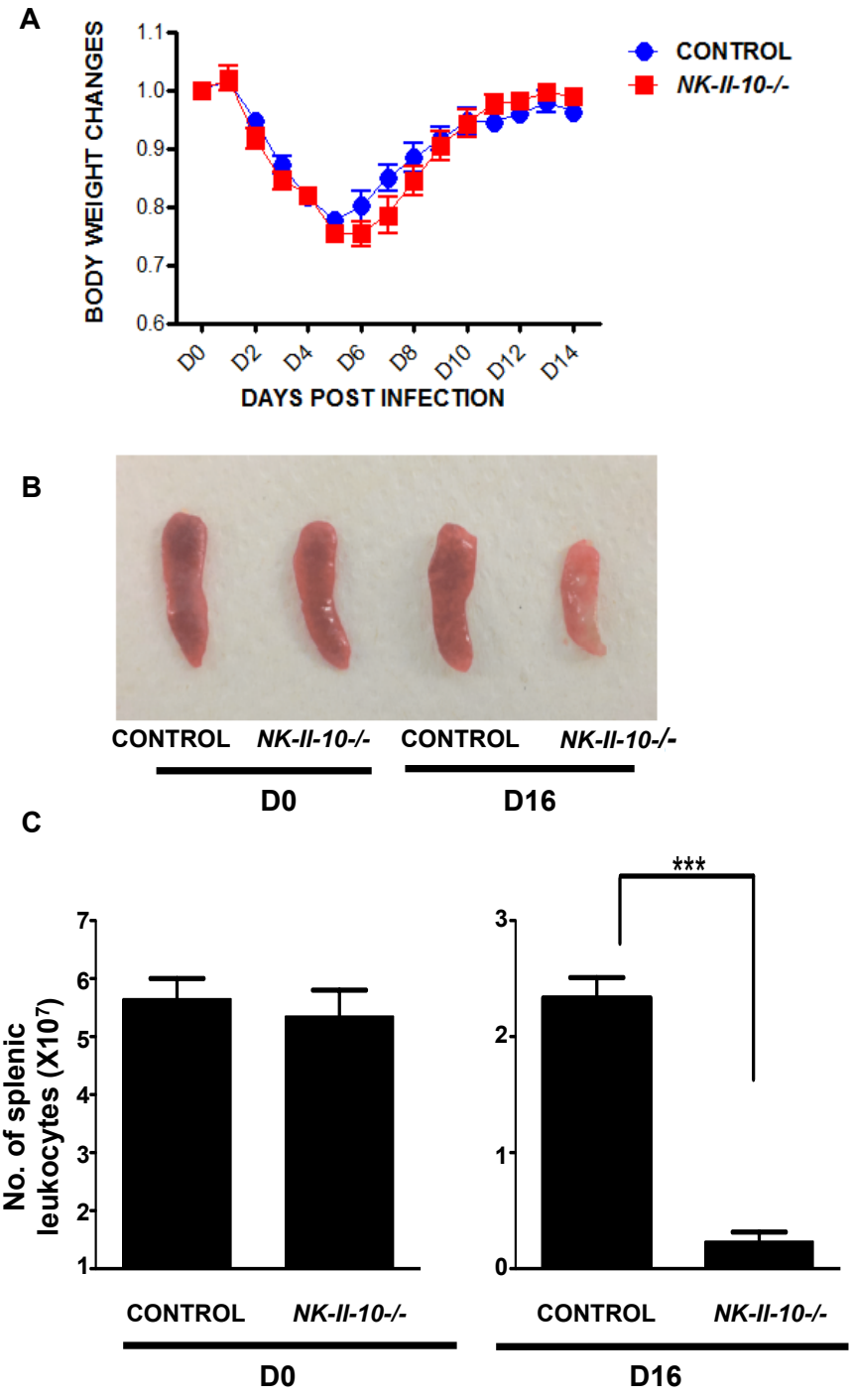

CD4+ T Cells
CD8+ T Cells
NK Cells
D0
Control
9
3
<1
<1
<1
85
NK-Il-10-/-
11
3
<1
<1
<1
79
D4
Control
22
31
31
51
67
89
NK-Il-10-/-
19
32
50
66
85
94
CD25
CD43
CD25
CD43
CD25
CD43

Figure 3.4.2 *NK-Il-10-/-* mice are highly susceptible to chronic MCMV infection. A) The body weight is measured till D14 of MCMV infection (n=3, 5 × 10^4 PFU/mice). Both the groups showed similar trend in body weight changes during MCMV infection. **B)** Spleen morphology of control and *NK-Il-10-/-* mice on D16 of MCMV infection is shown in the figure. Enhanced immunopathology in spleen of *NK-Il-10-/-* mice is observed after MCMV infection (n=3, 5 ×10^4 PFU/mice). C) The splenic lymphocytes number is determined in control and *NK-Il-10-/-* mice after MCMV infection. Severe reduction in splenic lymphocyte number is observed in *NK-Il-10-/-* mice. Statistical significances between groups are indicated (***p<0.001).


A
BODY WEIGHT CHANGES
1.1
1.0
0.9
0.8
0.7
0.6
D0
D2
D4
D6
D8
D10
D12
D14
DAYS POST INFECTION
CONTROL
NK-Il-10-/-
B
CONTROL
NK-Il-10-/-
CONTROL
NK-Il-10-/-
D0
D16
C
No. of splenic leukocytes (X10⁷)
7
6
5
4
3
2
1
***
3
2
1
0
CONTROL
NK-Il-10-/-
CONTROL
NK-Il-10-/-
D0
D16

control and *NK-Il-10-/-* mice. Interestingly, I observed significantly reduced lymphocyte number in spleen of *NK-Il-10-/-* mice with regard to control mice, suggesting severe lymphopenia in *NK-Il-10-/-* mice **(Figure 3.4.2C)**. Although the control group also displayed a slight reduction in lymphocyte number after infection, *NK-Il-10-/-* mice showed dramatic lymphocyte loss after infection. Such reduction in lymphocyte number and spleen necrosis can be considered as signs of severe immune mediated pathology during MCMV infection (Benedict et al., 2006; Bukowski et al., 1984; Daniels et al., 2001). All together, my data clearly indicate that mice lacking NK derived IL-10 are highly susceptible to disease mediated immune pathology.

3.4.3 MCMV persistence in salivary glands in the absence of NK cell derived IL-10

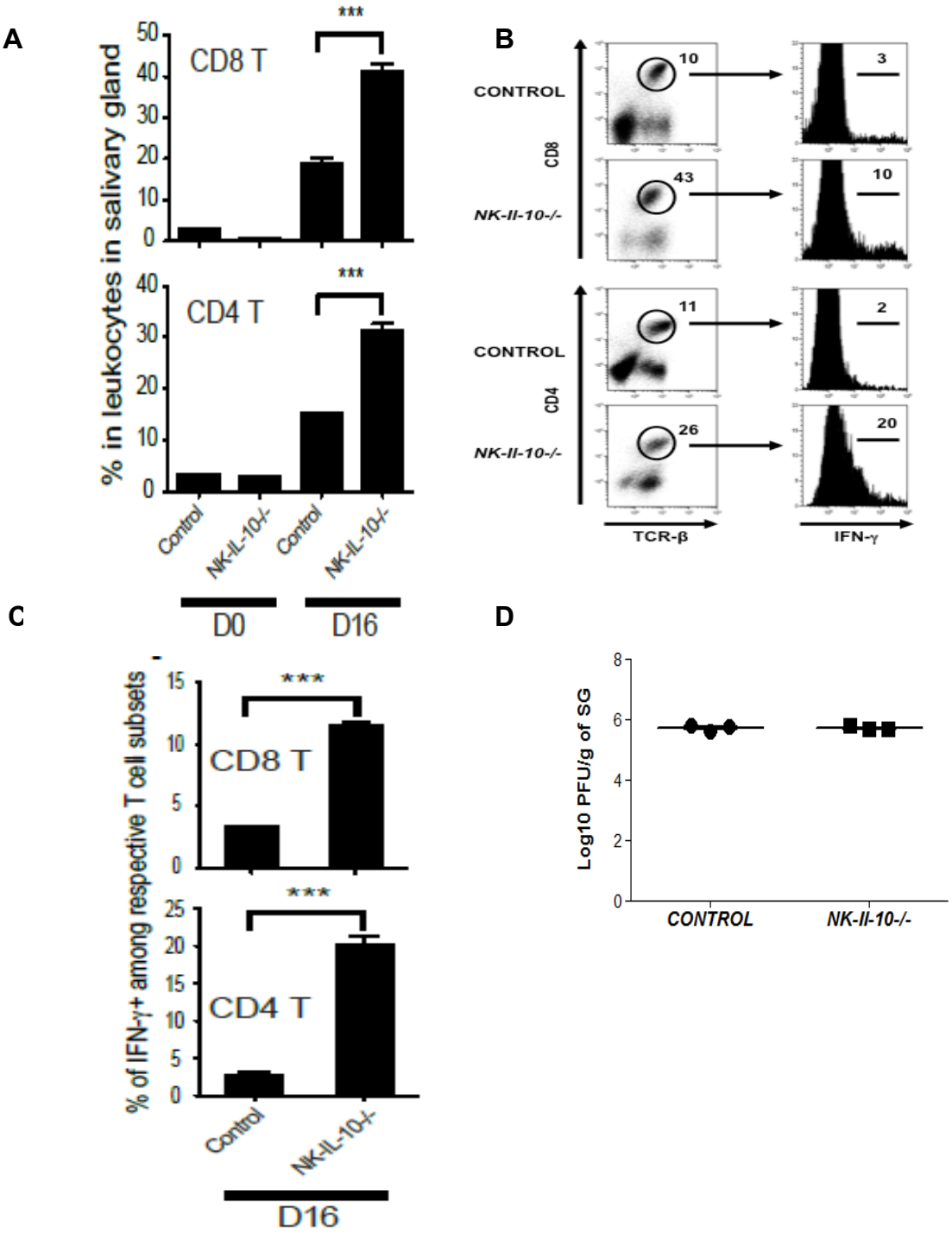
The salivary gland environment is highly immunosuppressive making it an immune privileged site for viral persistence (Huntington et al., 2007). Both MCMV and HCMV are known for establishing a lifelong latent infection inside the salivary glands and therefore act as natural source of horizontal viral transmission (Landolfo et al., 2003). Nevertheless, very little is known about the latent infection and type of immune response governing latency inside the salivary gland. The IFN-γ producing CD8+ T cells can clear MCMV efficiently from all other organs except submandibular glands (SMG) of salivary glands where it persists for longer period of time. Several studies have reported a role for IL-10 in maintaining the immunosuppressive environment inside the salivary glands (Humphreys et al., 2007; Mandaric et al., 2012). To study the effect of NK cell derived IL-10 in maintaining immunosuppressive environment and viral persistence inside salivary glands; I infected control and *NK-Il-10-/-* mice with 5×10^4 PFU of MCMV and analyzed the T cell response

in salivary glands on D16 p.i. Notably, I observed significantly higher proportion of CD4+ and CD8+ T cells in salivary glands of *NK-Il-10-/-* mice as compared to control mice on D16 of infection **(Figure 3.4.3A).** Moreover, I analyzed the percentage of IFN-γ producing CD4+ and CD8+ cells on D16 by flow cytometry. I observed significantly higher IFN-γ producing CD8+ in *NK-Il-10-/-* mice as compared to control mice **(Figure 3.4.3B)**. Also, the proportion of IFN-γ producing CD4+ T cells was significantly higher in *NK-Il-10-/-* mice in regard to control mice **(Figure 3.4.3B)**. The summarized flow data in **Figure 3.4.3C** also depicted an increased proportion of IFN-γ producing CD4+ and CD8+ cells in *NK-Il-10-/-* mice. Even though**,** the proportion of IFN-γ producing CD4+ T cells is higher than CD8+ T cells but CD8+ T cells are more potent in effector functions than CD4+ T cells. One would expect increased viral clearance and subsequently reduced viral burden in salivary glands of *NK-Il-10-/-* mice because of the enhanced IFN-γ response. However, I observed comparable viral burden in salivary glands of control and *NK-Il-10-/-* mice on D16 **(Figure 3.4.3D)**. Taken together, my data indicated enhanced CD4+ and CD8+ T cell response in salivary glands in the absence of NK cell derived IL-10 suggesting its contribution to immunosuppressive environment of salivary glands and potential role in viral persistence. Nonetheless, I did not observe enhanced MCMV control in *NK-Il-10-/-* mice, suggesting the complex nature of immunosuppressive environment in the salivary glands.

3.5 Regulatory role of NK cells during LM infection

To determine whether the NK cell-mediated immunoregulation can be extended to bacterial infections, I sought to study the regulatory role of NK cell derived IL-10 during chronic LM infection. I infected control and *NK-Il-10-/-* mice with 2×10^4 CFU and

Figure 3.4.3 NK cell derived IL-10 modulates T cell response in salivary gland during MCMV infection. A) Proportion of CD4+ and CD8+ T cells in salivary glands D16 after MCMV infection (n=3, 5 × 10^4 PFU/mice) is determined by flow cytometry. A significant increase in percentage of CD4+ and CD8+ T cells is observed in salivary glands of *NK-Il-10-/-* mice. Statistical significances between groups are indicated (*** p≤ 0.001). **B)** The IFN-γ production by different T cell subsets is determined by flow cytometry. Significantly higher IFN-γ production was observed in CD4+ T and CD8+ T cells upon stimulation with α-CD3 + α-CD28 D16 after MCMV infection (n=3, 5 × 10^4 PFU/mice). C) Summary of flow cytometric data in graphical form representing IFN-γ production by different T cell subsets. Statistical significances between groups are indicated (*** p≤ 0.001). **D)** The viral titer was assessed in salivary glands of control and NK-Il-10-/- mice on D16 post MCMV infection (n=3, 5 × 10^4 PFU/mice). There is no difference in salivary gland viral burden of both the groups.


A
B
C
D
CD8 T
CD4 T
% in leukocytes in salivary gland
***
Control
NK-IL-10-/-
D0
D16
CONTROL
NK-Il-10-/-
CD8
CD4
TCR-β
IFN-γ
10
3
43
10
11
2
26
20
% of IFN-γ+ among respective T cell subsets
Log10 PFU/g of SG
CONTROL
NK-Il-10-/-

measured the body weight for 14 days. Notably, *NK-Il-10-/-* mice lost significantly more body weight as compared to control mice indicating increased susceptibility of mice lacking NK derived IL-10 during LM infection **(Figure 3.5.1)**. The striking difference in body weight among control and *NK-Il-10-/-* mice became evident around D4 with maximum drop observed on D5 of infection. The control group recovered pre-infection body weight by D14 however the *NK-Il-10-/-* mice still displayed lower body weight than their pre infection weight. Interestingly, at every time point after D5, *NK-Il-10-/-* mice were found to be recovering slowly as compared to control mice, indicating that mice lacking early IL-10 production by NK cells are highly susceptible to acute LM infection. In order to determine the effect of NK derived IL-10 on the activation status of T cells, I infected control and *NK-Il-10-/-* mice with 2.5×10^4 CFU of LM and analyzed the expression of activation markers, CD43 and CD25 on CD8+ T cells and NK cells at D4 p.i. Unlike MCMV infection, I didn't observe differences in expression of CD43 and CD25 on the surface of CD8+ T and NK cells **(Figure 3.5.2A)**. Based on the maximum drop in body weight around D4-D5, I determined the bacterial burden in spleens from control and *NK-Il-10-/-* mice at D4. I observed comparable bacterial burden between both the groups **(Figure 3.5.2B)**.

Altogether, I obtained two conflicting conclusions from my LM data. The difference in body weight between control and *NK-Il-10-/-* mice suggested a possible regulatory role of NK cell derived IL-10 in the early stages of LM infection. It might be possible that absence of NK cell derived IL-10 during these early phases of infection results in highly activated immune response resulting in enhanced immunopathology as observed by the severe drop in body weight around D4-D5. On the other hand, comparable activation status of CD8+ T cells and bacterial burden in the spleen suggested no role of NK cell derived IL-10 in modulating

Figure 3.5.1 NK cell derived IL-10 protect host from excessive disease associated pathology during LM infection. The body weight is measured till D14 of LM infection**.** *NK-Il-10-/-* mice lost significantly more weight as compared to control mice after LM infection (n=3 for control and n=9 for *NK-Il-10-/-* mice, 2×10^4 CFU/mice). Statistical significances between groups are indicated (**p≤ 0.01).

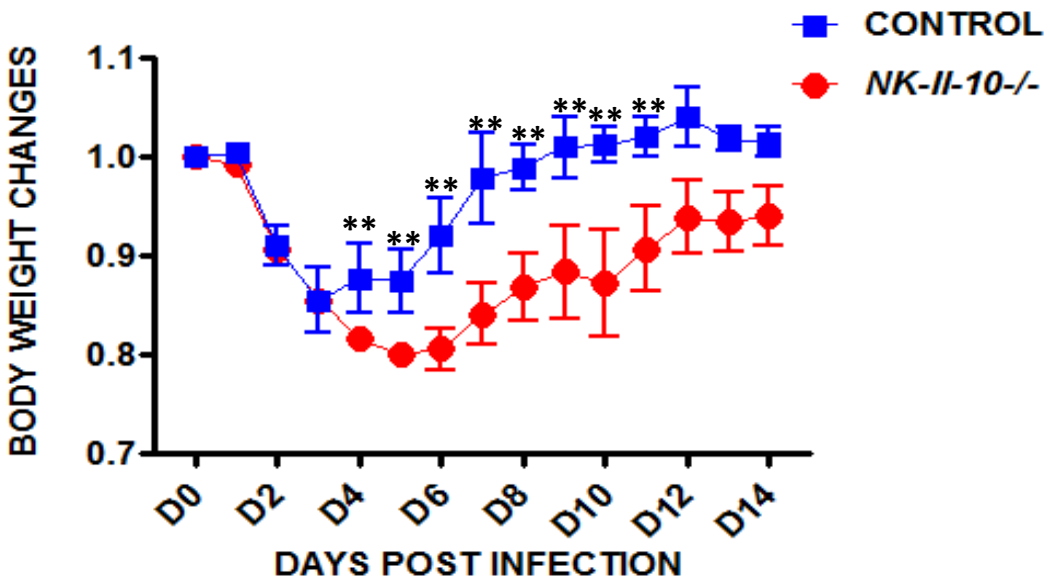
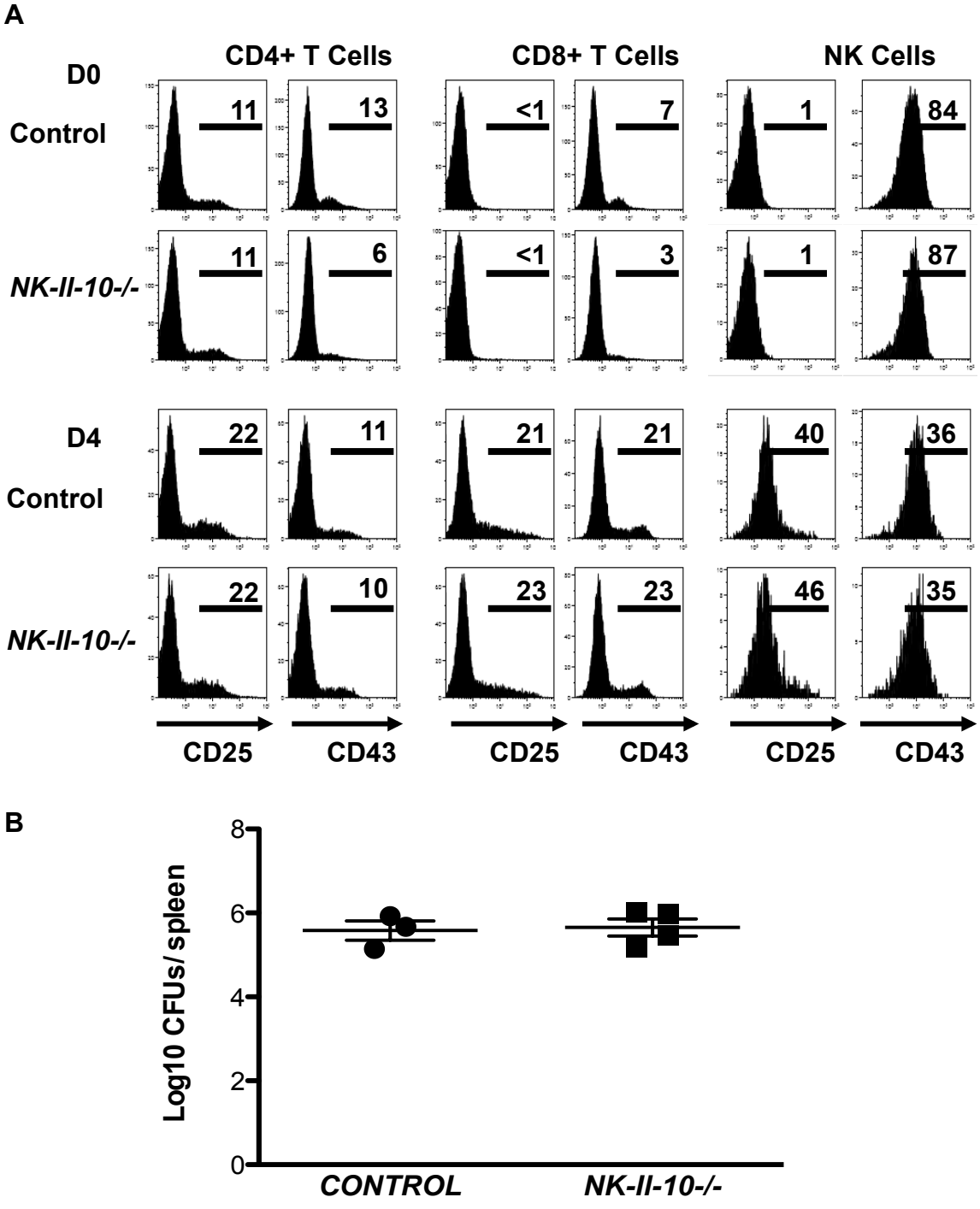

CONTROL
NK-Il-10-/-
BODY WEIGHT CHANGES
1.1
1.0
0.9
0.8
0.7
D0
D2
D4
D6
D8
D10
D12
D14
DAYS POST INFECTION
**
**
**
**
**
**
**
**

Figure 3.5.2 NK cell derived IL-10 has negligible effect on T cell response during LM infection. A) The expression of activation markers CD43 and CD25 on different cell subsets in spleen of control and *NK-Il-10-/-* mice is determined by flow cytometry. No difference in expression of activation markers on CD8+ T cells and NK cells is observed among control and *NK-Il-10-/-* mice is observed on D4 post LM infection (n=3 for control and n=4 for *NK-Il-10-/-* mice, 2.5×10^4 CFU/mice). **B)** The bacterial burden is estimated on D4 by colony assay. Both the groups displayed comparable bacterial burden (n=3 for control and n=4 for *NK-Il-10-/-* mice, 2.5×10^4 CFU/mice).


A
CD4+ T Cells
CD8+ T Cells
NK Cells
D0
Control
11
13
<1
7
1
84
NK-Il-10-/-
11
6
<1
3
1
87
D4
Control
22
11
21
21
40
36
NK-Il-10-/-
22
10
23
23
46
35
CD25
CD43
CD25
CD43
CD25
CD43
B
Log10 CFUs/ spleen
8
6
4
2
0
CONTROL
NK-Il-10-/-

the overall immune response during LM infection. In my opinion, the absence of early regulatory NK function might result in rapid bacterial spread accompanied by enhanced inflammation (indicated by increased body weight loss in *NK-Il-10-/-* mice) during early phases but as infection progresses the enhanced inflammation might induce other immune cells to produce more IL-10 for compensating the missing early regulatory function. This compensatory phenomenon might results in enough accumulation of IL-10 that subsequent T cell response is comparable in both the groups and that's why I did not observe difference in activation status of T cell after infection.

4. Discussion

4.1 A brief summary

NK cells have been well recognized as potent pro-inflammatory cells due to their capability of secreting cytokines such as, IFN-γ and TNF-α that limit microbial growth during the initial stages of infections (Biron et al., 1999; Lee et al., 2007). Furthermore, NK cell derived IFN-γ helps to shape the T cell response in the lymph nodes, possibly by direct interaction between naïve T cells and NK cells migrating from the site of inflammation to secondary lymphoid organs or by an indirect effect on DCs (Martin-Fontecha et al., 2004). Notably, recent reports have demonstrated IL-10 production by NK cells suggesting their role in regulating the immune response (De Maria, 2007; Lee et al., 2009). In this study, I have demonstrated that NK cells are a key source of IL-10 during early phases of MCMV and LM infection and therefore, may have a role in regulating the overall immune response. In my experiments, the proportion of IL-10 GFP expressing NK cells during LM infection was different from what was documented by Perona-Wright et al (Perona-Wright et al., 2009). The use of a different IL-10 GFP reporter mouse and LM strain for infection in the previous study might account for the observed differences in IL-10 GFP expression.

I generated *NK-Il-10-/-* mice to elucidate the regulatory role of NK cells during microbial infections. I confirmed the IL-10 deficiency in NK cells at the genomic and protein level. Whether spontaneous intestinal inflammation develops in the *NK-Il-10-/-* mice was intriguing because both conventional *Il10*-/- mice and T cell-specific *Il-10-/-* mice on the B6 background develop rectal prolapse and diarrhea that are indicators of severe spontaneous intestinal inflammation at 6 months of age (Kuhn et al., 1993). Although T_{regs} have been

known to play a central role in suppressing immune mediated inflammation, it is possible that additional IL-10 sources are required to restrain spontaneous gut inflammation. My analysis of colon histology showed absence of any aberrant intestinal inflammation in *NK-Il-10-/-* mice, indicating that NK cell derived IL-10 is not required for maintaining gut homeostasis. Taken together, the *NK-Il-10-/-* mouse is a useful tool to study regulatory role of NK cells and can be employed in various infection studies.

To dissect the immunoregulatory role of NK cells during infection, I decided to use MCMV and LM infection models. First, I infected *NK-Il-10-/-* and control mice with MCMV and observed severe lymphopenia and discoloration of spleens in *NK-Il-10-/-* mice as compared to control littermates. The severe discoloration and reduction in lymphocyte number may be due to increased activation-induced apoptosis driven by pro-inflammatory cytokine in the absence of IL-10, as observed in one of the recent study with NK cells during acute MCMV infection where anti–IL-10R blockade increased the frequency of NK cell apoptosis with cells exhibiting a phenotype indicative of activation-induced cell death (Stacey et al., 2011). The role of cytokines and chemokine in mediating immune mediated pathology in infected organs is also supported by a previous study conducted to characterize inflammatory response in MCMV infected liver. This study demonstrated enhanced NK cell dependent migration of monocyte/macrophage to site of infection. They also observed elevated IFN-γ production suggesting the role of pro-inflammatory cytokines in mediating pathology at the site of infection (Salazar-Mather et al., 1998). It is conceivable that IL-10 being a potent regulatory cytokine can protect the organ architecture from the immune mediated pathology by limiting the production of pro-inflammatory cytokines. Hence, it would be interesting to see histological changes in spleen and liver at different time points

following MCMV infection. The production of IL-10 by other cell types such as macrophages, dendritic cells and T_{regs} may account for the similar body weight observed in control and *NK-Il-10-/-* mice upon MCMV infection. Interestingly, in contrast to MCMV, I observed a significant reduction in body weight of LM infected *NK-Il-10-/-* mice as compared to control mice. Nevertheless I did not observe a difference in the activation status of T cells in LM infected *NK-Il-10-/-* mice.

The loss of IL-10 function either by genetic deletion of *Il-10* gene or blocking IL-10 receptor result in the accumulation of IFN-γ producing CD4+ T cells and reduced viral burden suggesting a role of IL-10 in maintaining an immunosuppressive environment inside the salivary glands (Humphreys et al., 2007; Mandaric et al., 2012). Therefore it is possible that IL-10 produced by NK cells is also contributing to immunosuppressive environment in salivary gland. Moreover, a recent paper demonstrated IL-10 production by NK cells in salivary glands on D10 of MCMV infection. They observed an unusual phenotype of NK cells being hyporesponsive but at same time expressing high level of activation markers such as KLRG1 and CD69 (Tessmer et al., 2011). Altogether, their data imply that MCMV latency in salivary gland could result from inadequate NK cell responses. Notably, I observed a significantly higher proportion of CD4+ and CD8+ T cells in salivary glands of *NK-Il-10-/-* mice as compared to control mice during the latent stage of MCMV infection.

In addition, the proportion of IFN-γ producing CD8+ and CD4+ T cells was significantly higher in *NK-Il-10-/-* mice as compared to control mice suggesting that NK cell derived IL-10 also contributes to the immunosuppressive environment of salivary gland. Due to enhanced T cell activity observed in salivary glands of *NK-Il-10-/-* mice, I expected to have decreased viral burden but contrary to my expectation, I observed comparable viral load

in both the groups. Taken together, NK cell derived IL-10 is important in protecting the host from excessive disease mediated pathology and in regulating the T cell response. Nonetheless, I failed to see enhanced virus control translated from the unleashed T cell response, suggesting the presence of compensatory pathways mediated by other IL-10 producing cells.

4.2 IL-10: a potent immunosuppressive cytokine

IL-10 is a pleotropic immune regulatory cytokine that suppresses T cell responses primarily via effects mediated on APCs. Specifically, IL-10 inhibits the expression of pro-inflammatory cytokines and chemokines, MHC, and co-stimulatory molecules by APCs (Moore et al., 1993; O'Garra et al., 2008). IL-10 has paradoxical functions during viral infections. For example, in acute influenza infection, blocking the action of the T cell-derived IL-10 results in enhanced pulmonary inflammation and lethal injury (Sun et al., 2009). Additionally, IL-10 blockade during acute herpes simplex infection resulted in severe disease and enhanced stromal keratitis suggesting a role of IL-10 in limiting disease-mediated pathology (Sarangi et al., 2008). The protective role of IL-10 in limiting excessive inflammation has also been observed during acute respiratory syncytial virus (RSV) infection. RSV infection of IL-10-deficient mice resulted in increased weight loss and an enhanced RSV-induced CD8+ and CD4+ T cell response that correlated with increased disease severity in the absence of IL-10 or following IL-10R blockade (Weiss et al., 2011). The studies with acute MCMV infection of total IL-10 knockout mice showed increased body weight loss, reduced viral titers and prolonged pro-inflammatory cytokine production by T cells (Mandaric et al., 2012; Oakley et al., 2008). All together, these studies

demonstrate that IL-10 plays a critical role in modulating the adaptive immune response by limiting T-cell-mediated inflammation and injury.

On the contrary, IL-10 can interfere with protective immunity following a high-dose influenza challenge by inhibiting Th1 and Th17 responses whereas blocking IL-10 can protect naïve mice against an otherwise lethal influenza challenge (McKinstry et al., 2009). Furthermore, during persistent LCMV infection, the suppressive effect of IL-10 was abrogated upon genetic removal of *Il-10* resulting in the maintenance of robust effector T-cell responses, the rapid elimination of virus and the development of antiviral memory T-cell responses (Brooks et al., 2006). The IL-10 production by CD4+ T cells limits the T cell immune response in salivary gland whereas blocking of IL-10 receptor with an antagonist antibody dramatically reduces the viral load in the salivary glands suggesting a role of IL-10 in contributing to immunosuppression and MCMV persistence inside the salivary gland (Humphreys et al., 2007). In addition, IL-10 is also thought to contribute to the immune suppressive environment inside the tumor. However, some recent studies demonstrate that IL-10 also possesses strong antitumor activity (Mumm et al., 2011; Tanikawa et al., 2012). Taken together, IL-10 can be beneficial to the host by protecting from excessive disease-mediated immunopathology and increasing tumor immune surveillance or it can be detrimental to host by inducing immunosuppression and thereby allowing pathogen persistence. It is noteworthy to mention that the increased accumulation of IFN-γ producing CD4+ and CD8+ T cells observed in salivary glands *NK-Il-10-/-* mice during high dose MCMV infection indicated the role of NK cell derived IL-10 in dampening the overall T cell response and thereby resulting in virus persistence. But as mentioned previously, IL-10 interferes with antigen presentation; it would be interesting to look at the MHC expression

on macrophages and DCs during MCMV infection in *NK-Il-10-/-* mice. In my opinion, early IL-10 production by NK cells has wide applications in modulating inflammation for favorable consequences during various diseases. For example, blocking NK regulatory pathway can induce effective T cell responses against tumors and chronic infections. On the other hand, inducing NK cell regulatory pathway during autoimmune diseases, transplantation-related disorders, and infections with acute inflammation can help restrain excessive disease mediated inflammation.

4.3 CMV IL-10 homologs (cmvIL-10): a strategy to escape immune response

Over the millions of years of coevolution with their hosts, viruses have developed several strategies to evade the host immune system. One of these strategies is to express immune modulators encoded within viral genomes. Viruses use homologs of cellular cytokines or cytokine receptors to shield infected cells from immune defenses and enhance their survival in the host. The presence of virus-encoded homologs of cellular proteins may be an indicator of the importance of these cellular components in immune mechanisms for combating this virus *in vivo*. A number of herpes viruses harbor homologs of IL-10 suggesting a role of IL-10 in modulating local immune response so as to enhance the capacity to replicate, disseminate, and/or persist in an otherwise immunocompetent individual. Whether in acute, persistent, or latent infection settings, the expression of virus-encoded IL-10 homologs would serve as an efficient means to interfere with multiple immune components to ensure successful infection of the host. The first viral homolog of IL-10 was identified in Epstein–Barr virus (EBV) (ebvIL-10) and it shares most of the biological activities with cellular IL-10 (Moore et al., 1990). In addition to EBV, another virus, the Orf poxvirus (OV), which can infect humans, has its own IL-10 homolog, ovIL-10

(Fleming et al., 1997). Similar to other herpes viruses, HCMV also expresses a functional IL-10 homolog (cmvIL-10) indicating the importance of IL-10 in the suppression of HCMV-specific immunity. An open reading frame UL111a encodes for cmvIL-10 homolog. It demonstrates approximately 27% identity to human IL-10 based on comparison of amino acid sequences, in contrast to ebvIL-10 that is almost 80% identical. Despite very low similarity, cmvIL-10 can bind to the IL-10 receptor complex and is capable of inducing signal transduction. The presence of the first two introns of cmvIL-10 gene at position similar to first and third intron of human IL-10 suggests that these genes are related. It also suggests that HCMV might have acquired partially spliced IL-10 mRNA sequence from infected cells. Alternatively, CMV might have captured the human IL-10 genes that subsequently evolved to eliminate two introns and shorten the remaining two. (Kotenko et al., 2000; Slobedman et al., 2009).

The cmvIL-10 possesses immunosuppressive properties. The *in vitro* study with both the purified bacterium derived recombinant cmvIL-10 and cmvIL-10 collected from the supernatants of human cells showed inhibited proliferation of peripheral blood mononuclear cells (PBMCs), production of pro-inflammatory cytokines IL-1α, IL-6, granulocyte-macrophage colony-stimulating factor and TNF-α in LPS treated PBMCs and monocytes. Additionally, cmvIL-10 was observed to reduce the surface expression of MHC molecules whereas it enhanced the expression of non-classical MHC class I molecule HLA-G. The presumed function of HLA-G is to confer the protection from NK cell mediated lysis of MHC class I negative cells (Spencer et al., 2002). The cmvIL-10 can skew monocyte differentiation towards a more phagocytic phenotype and away from the antigen presentation phenotype (Jaworowski et al., 2009). Another recent study demonstrated that in addition to

binding to the IL-10Rs, cmvIL-10 significantly up-regulates the production of endogenous cellular IL-10 by LPS-activated DCs. Thus, HCMV may subvert the degree and quality of DCs activated by local virus replication through the expression of cmvIL-10 homologue and induction of endogenous cellular IL-10 production from the cells that are not directly infected by the virus (Chang et al., 2009). Intriguingly, *in vitro* experiments have demonstrated cmvIL-10 mRNA expression during latent HCMV infection suggesting a possible role of cmvIL-10 in inhibiting recognition of latently infected cells by the memory CD4+ T cell (Cheung et al., 2009; Jenkins et al., 2004). All together, characterization of biological functions conserved by cmvIL-10 contributes to our knowledge of the virus-host interactions and also provides greater understanding of the important targets to limit persistent virus replication in mucosal tissues such as salivary glands, which in turn may block transmission of virus to new hosts.

4.4 Regulatory NK cells: functions in pregnancy, autoimmunity and immunoediting

The NK cells are one of the predominant populations of lymphocytes found during early gestation. The human uterine NK cells (uNK) have also been shown to produce IL-10 via IL-2/IL-12 mediated stimulation. The current speculation about uNK derived IL-10 during pregnancy is the protection of fetus from harmful maternal immune response by inhibiting production of deleterious pro-inflammatory cytokines (Vigano et al., 2001). In addition to shaping T-cell responses (mentioned earlier), NK cells can, however, eliminate activated autoreactive T cells and ameliorate experimental autoimmune encephalitis (EAE) (Winkler-Pickett et al., 2008). Following activation, T cells up-regulate NKG2D ligands and become susceptible to autologous NK cell mediated cytotoxicity (Cerboni et al., 2007). In this context, Lu et al. demonstrated that the interaction between the mouse homologue of the

human MHC class I molecule HLA-E, Qa-1-Qdm, on activated T cells and CD94/NKG2A inhibitory NK cell receptors, protects activated CD4+ T cells from perforin-mediated NK-cell cytotoxicity. Blocking this interaction by using anti-Qa-1 antibodies resulted in a potent NK cell mediated elimination of activated autoreactive T cells (Lu et al., 2007). Thus, NK cells contribute to the resolution of adaptive immune responses during autoimmune disorders via deletion of activated T cells.

Furthermore, NK cells might play an important regulatory role by selectively editing APCs during the course of immune responses. The activated NK cells can kill autologous immature myeloid DCs via NKp30-, NKp46-, and DNAM-1–mediated recognition suggesting that NK cells are important in editing non-immunogenic DCs (Spaggiari et al., 2001). Similarly, activated NK cells via NKG2D- and NKP46-mediated recognition lyses human microglial cells, resident macrophage-like APCs of CNS (Lunemann et al., 2008). Up-regulating expression of class I MHC or molecules similar to MHC can achieve resistance to NK cell mediated lysis (Lunemann et al., 2009). This suggest that NK cells reduce the pool of immature DCs and resting microglial cells during immune activation, and allow fully activated APCs to present antigens to the infiltrating T cells and to initiate a limited immune response. Taken together, NK cells are multicompetent lymphocytes with the ability to regulate innate and adaptive immune responses through regulatory cytokine production and interaction with APCs as well as T cells and B cells. Therefore, investigating immunoregulatory NK cell functions will generate exciting insights into NK cell mediated modulation of innate and adaptive immune responses, improve our capacity to monitor their role in immune suppression and viral persistence, perhaps, provide new prospects for NK cell directed therapies.

5. CONCLUDING REMARKS

In conclusion, I have confirmed IL-10 production by NK cells during early stages of MCMV and LM infection and successfully generated *NK-Il-10-/-* mice that are free from aberrant inflammation and therefore are an ideal tool to elucidate the role of NK cell derived IL-10 during microbial infection. The proposed working model in **Figure 5.1** depicts the possible role of NK cell derived IL-10 during microbial infections and expected outcome of an immune response in the absence of it. The *NK-Il-10-/-* mice exhibited increased susceptibility against MCMV and LM infections. Moreover absence of NK cell derived IL-10 resulted in higher proportion of IFN-γ producing T cells in MCMV infected salivary glands. However, in spite of an enhanced immune response in infected *NK-Il-10-/-* mice, no improvement in viral clearance suggested the presence of other compensatory pathways for immune regulation. The brief summary of results from both MCMV and LM infection has been shown in **Table 5.1**. Taken together, NK cell derived IL-10, a double-edge sword: can be used as a prime target for immunotherapy to confer protection against disease-mediated pathology and in eliminating chronic infections. Furthermore, my study provides grounds to reassess the effect of NK cell derived IL-10 on T cell response, not only in the context of infectious diseases, but also in tumor immunology and transplantation.

FIGURE 5.1 Working model of regulatory role of NK cells during infections. Schematic showing role of NK cell derived IL-10 in regulating the immune response. **A)** The outcome of immune response in control mouse. The production of IL-10 by NK cells during early phases of infection inhibits pro-inflammatory cytokine production by other immune cells and also interferes with the activation of adaptive immune response. This early production of regulatory cytokine protects the host from excessive immune mediated pathology but may results in pathogen persistence. **B)** The expected outcome of immune response in *NK-Il-10-/-* mice. The loss of IL-10 function in NK cells may result in enhanced pathogen clearance but at an expense of excessive immune mediated pathology. **C)** The observed immune response in *NK-Il-10-/-* mice. The loss of IL-10 production by NK cells during early stages of infection results in higher inflammation other immune cells compensate for the missing IL-10 function resulting no improvement in pathogen clearance in *NK-Il-10-/-* mice. The solid black arrows represent the activation of immune cells and/or production of cytokines. The solid red lines represent the inhibition. The magnitude of cell responses is depicted in arbitrarily.

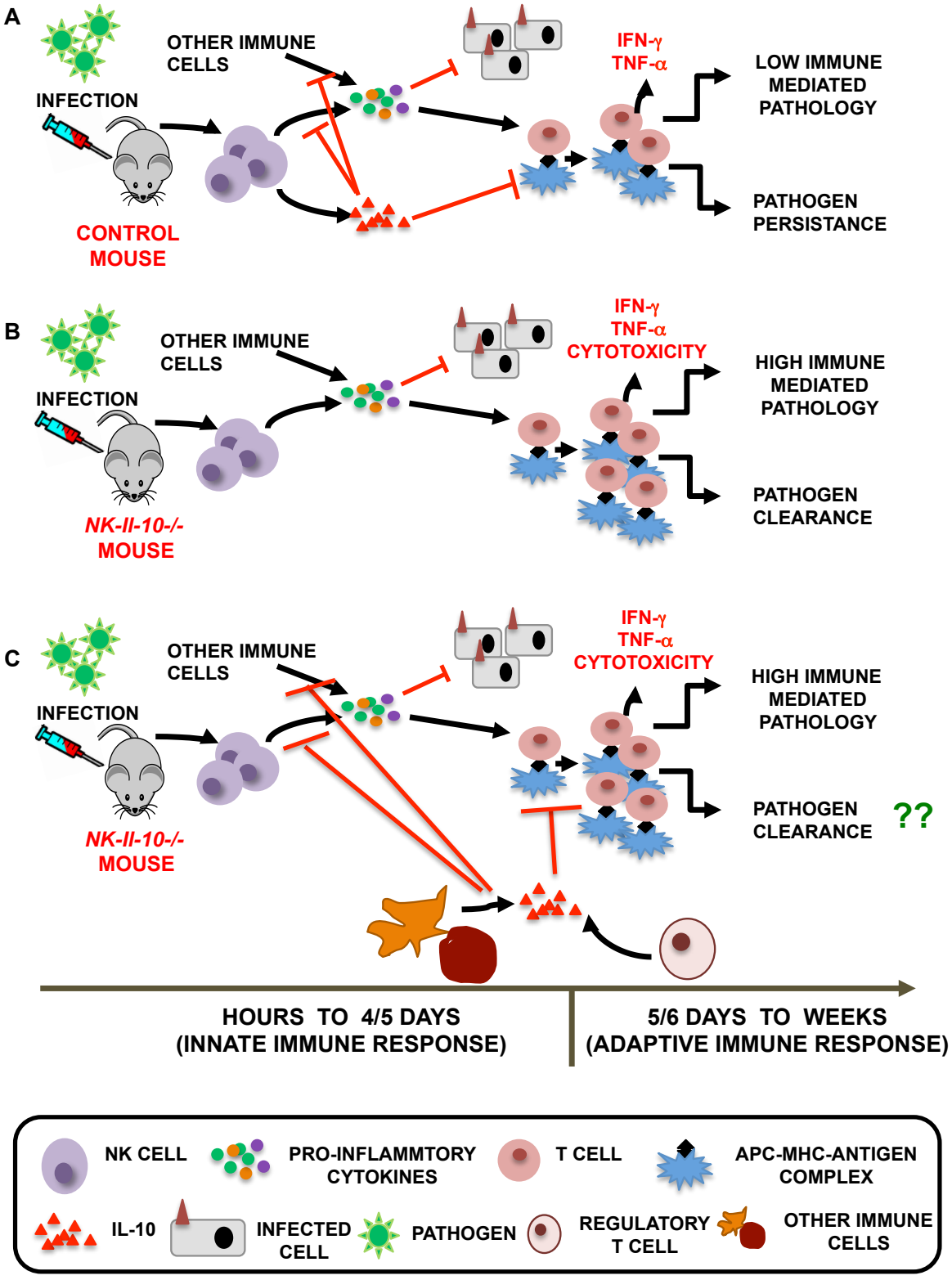

A
OTHER IMMUNE CELLS
INFECTION
CONTROL MOUSE
IFN-γ
TNF-α
LOW IMMUNE MEDIATED PATHOLOGY
PATHOGEN PERSISTANCE
B
OTHER IMMUNE CELLS
INFECTION
NK-Il-10-/- MOUSE
IFN-γ
TNF-α
CYTOTOXICITY
HIGH IMMUNE MEDIATED PATHOLOGY
PATHOGEN CLEARANCE
C
OTHER IMMUNE CELLS
INFECTION
NK-Il-10-/- MOUSE
IFN-γ
TNF-α
CYTOTOXICITY
HIGH IMMUNE MEDIATED PATHOLOGY
PATHOGEN CLEARANCE
??
HOURS TO 4/5 DAYS (INNATE IMMUNE RESPONSE)
5/6 DAYS TO WEEKS (ADAPTIVE IMMUNE RESPONSE)
NK CELL
PRO-INFLAMMTORY CYTOKINES
T CELL
APC-MHC-ANTIGEN COMPLEX
IL-10
INFECTED CELL
PATHOGEN
REGULATORY T CELL
OTHER IMMUNE CELLS

Table 5.1 Tables summarizing results from LM and MCMV infection. **A)** Results from IL-10 GFP reporter mice during LM and MCMV infection. NK cells are major producers of IL-10 during early stages of MCMV and LM infections. **B)** Results from control and *NK-Il-10-/-* mice during LM and MCMV infection. The *NK-Il-10-/-* mice exhibited increased susceptibility against MCMV and LM infections.

A) Results from IL-10 GFP mice

Parameter	Murine Cytomegalovirus	*Listeria Monocytogenes*
Infection dose Infection route	*1.2 × 10^4 PFU* i.p.	4 × 10^4 CFU i.v.
IL-10 production by NK cells	~80% NK cells were IL-10 expressing on D4 of infection	~30% NK cells were IL-10 expressing on D4 of infection
IL-10 production by CD4 T cells	~10% NK cells were IL-10 expressing on D4 of infection	~8% NK cells were IL-10 expressing on D4 of infection

B) Results from control and *NK-Il-10-/-* mice

Parameter	Murine Cytomegalovirus	*Listeria Monocytogenes*
Infection dose Infection route	5 ×10^4 PFU i.p.	2 × 10^4 CFU[a] 2.5 × 10^4 CFU[b] i.v.
Body weight change	Comparable in control and *NK-Il-10-/-* mice	*NK-Il-10-/-* mice lost significantly more weight as compare to control mouse
Spleen appearance	Comparatively smaller and discolored in *NK-Il-10-/-* mice as compare to control mouse	n.d.
Lymphopenia	Significantly lower number of leukocytes in *NK-Il-10-/-* mice as compare to control mouse	n.d.
Expression of CD43 and CD25 markers	Comparatively higher expression on CD8 T cells in *NK-Il-10-/-* mice than control mouse	Expression is comparable in *NK-Il-10-/-* mice and control mice

a- Infection dose used to measure body weight change

b- Infection dose used to measure expression of CD43 and CD25 markers

n.d.- not determined

i.p. intraperitoneal

i.v. intravenous

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Curriculum Vitae

Name: Amandeep Kaur Komal
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Citizenship: India
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Educational Background

M.Sc. in Microbiology and Immunology **2012-2014**
University of Ottawa, Ottawa, Ontario
Thesis: The immunoregulatory role of Natural Killer (NK) cell derived IL-10 during microbial infections.
Supervisor: Dr. Seung-Hwan Lee

M.Sc. in Biotechnology **2007-2009**
Punjabi University, Punjab, India
Thesis: To study the total phenolic content and antioxidant capacity of Green tea *(Camellia sinensis)*
Supervisor: Dr. Harpreet Kaur

Bachelor of Science - Biotechnology **2004- 2007**
Punjabi University, Punjab, India

Research Experience

University of Ottawa, Ottawa, Ontario **2012 - 2014**
M.Sc. in Microbiology and Immunology (Seung-Hwan Lee Lab)
Project: The immunoregulatory role of Natural Killer (NK) cell derived IL-10 during microbial infections.

- DNA extraction and genotyping of mice
- RNA extraction from cells, Real Time (RT)-PCR and qPCR analysis
- Intranasal, intravenous and intraperitoneal infection of mice
- In-Vitro cell culturing and maintenance, cell proliferation assays
- Cell enrichment by magnetic separation
- Surface and intracellular antibody staining of cells
- Cytokine analysis of tissue lysates and serum
- Enumeration of virus by plaque assay
- Enumeration of bacteria using colony forming units assay
- Multi-color flow cytometry
- Subcutaneous injection of tumor cells in mice and examining tumor growth
- Measurement of tumor size and analyzing tumor cells by flow-cytometer

Punjabi University, Patiala, Punjab, India **2007-2009**
M.Sc. in Biotechnology
Project: Phenolic content and antioxidant capacity of green tea (*Camellia sinensis*) and effects of adding milk on antioxidant activity

- Qualitative and quantitative determination of total phenolic content and antioxidant capacity of green tea by various biochemical methods
- Spectrophometry and thin layer chromatography
- Number Cruncher Statistical System (NCSS) analysis software

Bioage Training and R&D Center, Mohali, India **June-July 2008**
Upstream and downstream processing

Publications

Nandagopal, N., Ali, A.K., **Komal, A.K.** and Lee S.H. (2014). The critical role of IL-15-PI3K-mTOR pathway in Natural Killer cell effector functions. *Front. Immunol.* **5**:187. doi: 10.3389/fimmu.2014.00187

Scholarships and Awards

- **University of Ottawa, Ottawa, Canada** **2014**
  Dean's scholarship (Value $1000)

- **27th Annual Spring Meeting for the Canadian Society of Immunology**, **Quebec City, Quebec** **March 2014**
  Travel Award (Value $500)

- **University of Ottawa, Ottawa, Canada** **2012-2014**
  Full admission scholarship (Value $7500/year)

- **Seminar day, University of Ottawa, Ottawa** **2013**
  Third prize (Value $50)

- **Council of Scientific and Industrial Research and University Grants Commission (CSIR-UGC), New Delhi, India** **2009**
  Successfully completed National Eligibility Test (NET) for lectureship/junior Research Fellowship (JRF)

- **Punjabi University, Patiala, India** **2009**
  Second position in Masters in Biotechnology

- **Punjabi University, India** **2005**
  Third position in Annual science quiz competition

- **State level Scholarship, India** **2002**
  Obtained state level scholarship in high school

Poster Abstracts

- **Komal, A.K.,** Nandagopal, N. and Lee, S.H. Role of Natural Killer (NK) cells derived IL-10 during microbial infections. **27th Annual Spring Meeting for the Canadian Society of Immunology**, March 2014, Quebec City, Quebec.

- **Komal, A.K.,** Nandagopal, N. and Lee, S.H. Role of Natural Killer (NK) cells derived IL-10 during Murine cytomegalovirus (MCMV) infection. **63rd Annual conference for the Canadian Society of Microbiology**, June 2013, Ottawa, Ontario.

- **Komal, A.K.,** Yuxia, Bo. and Lee, S.H. Investigation of immunoregulatory role of Natural Killer cells during influenza virus infection. **BMI Poster Day**, May 2012, Ottawa, Ontario.

Seminar Presentations

Komal, A.K., and Lee, S. Role of Natural Killer cells and CD4+ T cells in maintaining constant level of IL-10 during Murine cytomegalovirus (MCMV) infection. **BMI Seminar Day**, March 2013, Ottawa, Ontario. (**3rd prize awarded-Microbiology and Immunology**)

Affiliations

- **Canadian Society of Immunology-Student member** **2013-present**
  University of Ottawa, Ottawa, Ontario

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